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Anti-PEG antibodies in experimental animal disease models and in patients treated with PEGylated proteins

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TABLE OF CONTENT

1. Abstract

English

Anti-PEG antibodies in experimental animal disease models and in patients treated with PEGylated proteins

A number of protein therapeutics have been conjugated with polyethylene glycol (PEG) in order to increase the serum half-life and to reduce immunogenicity of these proteins. Examples are PEG-asparaginase, PEG-interferons, PEG-filgrastim and PEG-adenosine deaminase. PEG is a hydrophilic, uncharged, inert, biocompatible synthetic polymer that has long been thought to be non-immunogenic. However, it was shown in recent reports that treatment with PEGylated protein therapeutics can lead to the development of anti-PEG antibodies in animal disease models and in patients. Those antibodies caused a rapid clearance of the PEGylated proteins from the circulation. Moreover, a considerable amount of anti-PEG antibodies in healthy donors was reported in some studies.

Based on these reports, we developed an analytical method based on a flow-cytometric approach to detect anti-PEG antibodies. Anti-PEG antibodies are detected by FACS analysis using polystyrene beads that are grafted with PEG and further conjugated with branched PEG reagent. We established the assay and used it for monitoring the development of anti-PEG antibodies in disease models in mouse and rat during preclinical studies of the new drug candidates that are developed by Baxter Innovations GmbH. Furthermore we showed, that our modification of the polystyrene beads with the additional PEGylation improved the assay in comparison to similar approaches.

Moreover we used this assay to screen healthy blood donors for the potential presence of “natural” anti-PEG antibodies. This study in healthy blood donors provide important information for the screening of patients to be included in clinical studies of the new drug candidates.

Zusammenfassung

Anti-Peg Antikörper in Tierversuchsmodellen und in Patienten die mit PEGylierten Proteinen behandelt werden

Polyethylenglykol (PEG) ist ein hydrophiles, ungeladenes, inertes, biokompatibles synthetisches Polymer, welches heutzutage in vielen Gebieten zum Einsatz kommt. Eines dieser Anwendungsgebiete ist die Konjugierung von PEG an Protein- Therapeutika, um deren Halbwertszeit im Serum zu erhöhen. Beispiele für solche Therapeutika sind PEG - Asparaginase, PEG - Interferone, PEG - Filgrastim und PEG - Deaminase. PEG wird seit Jahrzehnten eingesetzt und galt lange Zeit als nicht immunogen. Neue Studien in Tierversuchsmodellen zeigten jedoch, daß es durch Behandlung mit PEGylierten Proteinen zur Entwicklung von anti-PEG Antikörpern kommen kann, welche zu einer raschen Entfernung des PEGylierten Proteins aus der Blutzirkulation führten. Überdies wurde in einer Studie berichtet, daß ein weitaus größerer Anteil von gesunden menschlichen Plasmaspendern „natürliche“ anti-PEG Antikörper besitzt, als ursprünglich angenommen.

Ziel dieser Arbeit war es, eine auf Durchflusszytometrie basierende, analytische Methode zum Nachweis von anti-PEG Antikörpern, zu etablieren. Dieser Nachweistest wurde dann in verschiedenen präklinischen Studien für derzeitige Produktkandidaten der Baxter Innovations GmbH verwendet, um die potentielle Entwicklung von anti-PEG Antikörpern in verschiedenen Tierversuchsmodellen zu verfolgen. Anti-PEG Antikörper wurden mittels FACS Analyse über Polystyrol – Kügelchen nachgewiesen, an deren Oberfläche lineares sowie verzweigtes (branched) PEG konjugiert wurde.

Wir konnten zeigen, daß die Behandlung mit PEGylierten Proteinen im Tierversuchsmodellen zur Entwicklung von anti-PEG Antikörpern führen kann. Zusätzlich können wir bestätigen, daß der Prozentsatz an „natürlichen“ anti-PEG Antikörpern in gesunden humanen Plasmaproben weitaus höher ist, als ältere Studien vermuten ließen. Die vielleicht bereits

vorhandenen anti-PEG Antikörpern, sowie die potentielle Entwicklung von anti-PEG Antikörpern in Patienten nach Behandlung mit PEGylierten Proteinen, muß in Zukunft bei klinischen Studien mit PEGylierten Proteinen berücksichtigt werden. Außerdem konnten wir zeigen, daß unsere Modifikation der Polysterol – Kügelchen mit verzweigtem (branched) PEG eine Verbesserung der Empfindlichkeit des Tests im Vergleich zu ähnlichen Testverfahren darstellt.

2. Introduction

2.1 Coagulation System

Coagulation is an important part of the hemostasis process by which blood forms solid clots after a blood vessel gets injured. Damage to small blood vessels and capillaries frequently occurs. There are three basic mechanisms that promote hemostasis and the termination of the bleeding.

The first reaction after an injury is local vasoconstriction and compression of injured vessels, thus diminishing blood loss. There are several mechanism including endothelial cell nitric oxide and prostacyclin that promote blood fluidity by preventing platelet stasis and dilating intact blood vessels. Those factors are no longer existing at the site of vessel damage, thus leading to the second mechanism: the adherence of platelets. Platelets are cells that are derived from megakaryocytes. Blood normally contains 150,000 to 350,000 per micro liter (µl). If this value should drop much below 50,000/µl, there is a danger of uncontrolled bleeding. This is because of the essential role that platelets have in blood clotting.

The starting point is the attachment of the soluble protein von Willebrand factor to the subendothelial matrix. There it exposes multiple intrinsic binding sites for the platelet specific membrane Glycoprotein Ib (GPIb). This binding stimulates platelet activation. The platelets expose the Glycoprotein IIbIIIa binding site for further attachment of von Willebrand factor and fibrinogen. Furthermore platelets undergo degranulation and release Adenosindiphosphate (ADP), which attracts more platelets to the area, serotonin and thromboxane A2, which cause vasoconstriction and Calcium. Thus ADP and thromboxane A2 promote more platelet adhesion and therefore more ADP and thromboxane. The positive feedback promotes the formation of a platelet plug. Calcium is essential in the activation of the soluble proteins of the coagulation pathway, which is the

final hemostatic mechanism. The plug formed by the platelets is relatively unstable and needs to be supported by a stronger clot, which is the final goal of the coagulation pathway. It leads to the generation of thrombin (factor IIa), which can then convert soluble fibrinogen into fibrin, which forms a clot (Colman et al, Hemostasis and Thrombosis 2001).

Two coagulation pathways provide normal human hemostasis: the extrinsic and the intrinsic pathway, that converge on a single common final pathway resulting in a clot. They are playing an important role for regulation of coagulation and do differ in their trigger for activation.

2.1.1 The intrinsic pathway

All components of this pathway are found within the blood. The reactions take place on the membrane surface and are initiated by activation of the contact factors by a negatively charged surface – e.g. tissue material such as collagen on the exposed surface of a blood vessel. The first step of the cascade is the binding of coagulation factor XII (FXII), or Hageman factor, to a sub-endothelial surface exposed after injury. FXII is a serine protease produced by the liver that circulates in plasma as an inactive zymogen. At the same time a complex of prekallikrein and high molecular weight kininogen (HMWK) binds in close proximity to FXII to the surface leading to the activation of FXII by proteolytic cleavage of the native Hagemann factor into two chains (50 and 26 kDa). Both chains are still linked by a disulphide bond after cleavage. The light chain (26kDa) contains the active site and this molecule is known as activated factor XII (FXIIa) (Colman et al, Hemostasis and Thrombosis 2001).

Once activated, FXIIa stays in contact with the surface and activates factor XI (FXI) by proteolytic cleavage. This step requires Ca^{++} to proceed efficiently and again HMWK, which binds to factor XI and facilitates the activation process. Furthermore FXIIa cleaves prekallikrein to end up with a higher amount of kallikrein, which also cleaves FXII: a positive feedback control loop has been established.

Activated factor XI (FXIa) assembles on the surface in complex with factor VIII (FVIII). This complex needs calcium to stabilize certain structures on these proteins associated with their membrane-binding properties. Factor X (FX) is binding the complex and is activated to FXa. After the activation of factor X we are talking about the *Common Pathway*, because all following steps are equal for the extrinsic pathway.

Factor V (FV) interacts with factor Xa in the presence of calcium ions on a phospholipids membrane surface to accelerate the conversion of prothrombin (factor II) to thrombin by factor Xa. Thrombin is the enzyme that cleaves the soluble fibrinogen to monomer fibrin. Many fibrin molecules polymerize forming long fibres. Factor XIII promote bonding between the units of the polymer.

2.1.2 The extrinsic pathway

The extrinsic pathway is an alternative way to produce fibrin. It provides a very rapid response to tissue injury, generating activated factor X almost instantaneously, compared with the seconds, or even minutes, required for the intrinsic pathway to activate factor X. The main function of the extrinsic pathway is to augment the activity of the intrinsic pathway.

Activation of this pathway occurs through protein tissue factor (tissue thromoblastin or factor III) that is normally not localized within the blood stream. Tissue factor is found on the surface of almost all cells, particularly abundant in the cells of brain, lungs and placenta (Drake et al 1989). Once activated, tissue factor binds rapidly to factor VII, which is then activated to form a complex of tissue factor, activated factor VII, calcium and platelet phospholipids. This complex then rapidly activates factor X. After activation of FX the common pathway is proceeding, leading to the generation of fibrin and a more stable clot.

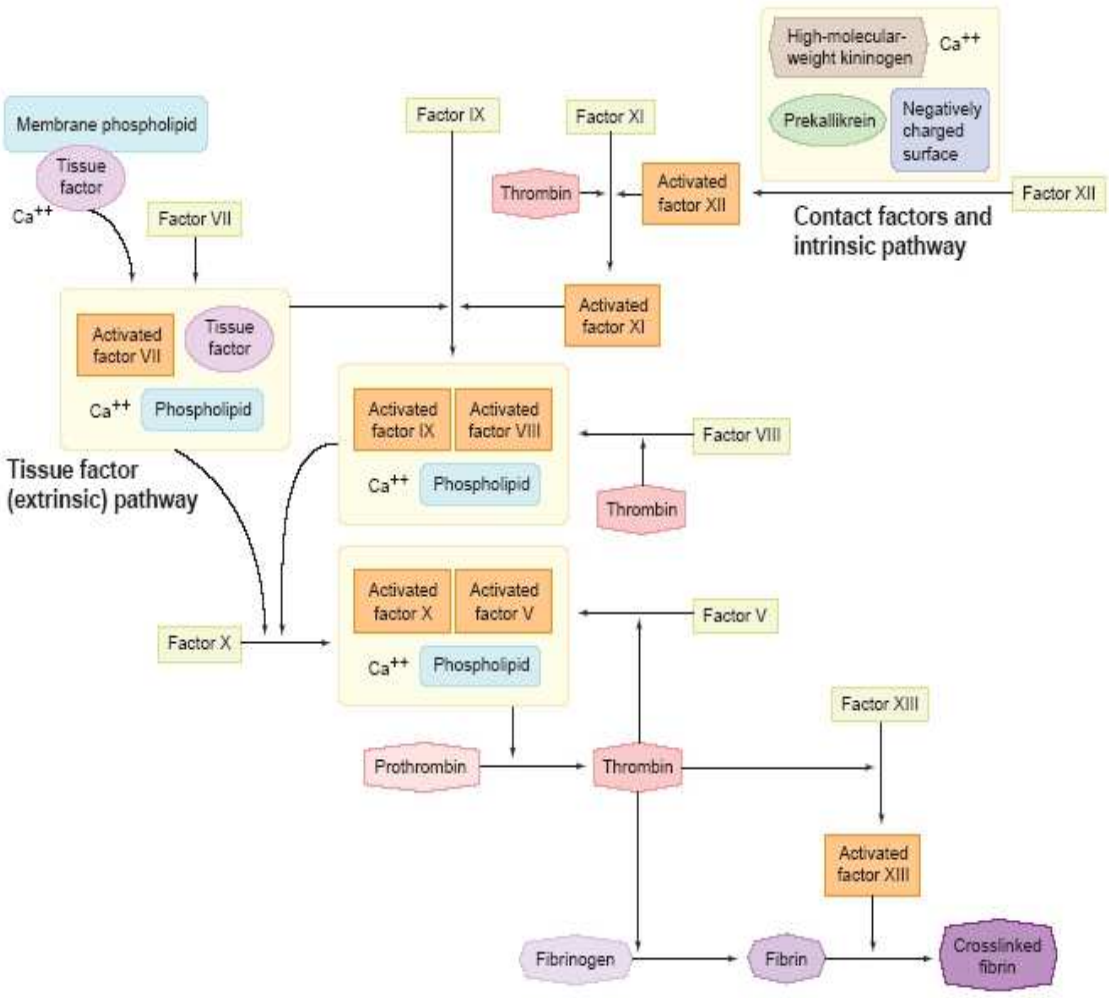

Figure 1: Coagulation pathway overview

Source: Dorland's Medical Dictionary for Health Consumers. © 2007 by Saunders, an imprint of Elsevier, Inc.

2.1.3 Biochemistry of blood coagulation

All proteins responsible for blood clotting after injury circulate in the blood as zymogens, which means they are in their inactive form. These precursor enzymes are cut at a specific bond that connects two amino acids within the polypeptide. The protein is split into specific fragments and an active enzyme is formed. This enzyme can split itself particular peptide bonds. This process is called limited proteolysis.

2.1.4 Regulation of blood coagulation

Blood clotting is life essential but the active enzymes must be turned off and the clotting process contained locally to the area of tissue injury. For this purpose we have a complex system of a series of blood proteins that assure the disengaging of the activated blood-clotting system.

For instance Antithrombin III is a plasma protein that binds most of the activated blood-clotting proteins in order to form inert complexes where the active site of the protease enzymes are inaccessible to its usual substrate. This action is greatly enhanced by the presence of heparin by inducing an allosteric change to antithrombin III. Heparin is a substance formed by mast cells of the connective tissue (Bjork I et al 1997)

Protein C, a vitamin K-dependent protein, is a zymogen that requires vitamin K for its activation by thrombin complexed to thrombomodulin, a protein on the endothelial cell membrane. Activated protein C is capable of inactivating the active cofactor forms of factors VIII and V. Its action is enhanced when bound protein S, a vitamin K-dependent protein that is attached to cell membranes (platelet or possibly endothelial cells). A deficiency in the level of protein C or protein S is associated with an excessive tendency to form clots (Griffin et al 2007)

Another anticoagulant effect is the fibrinolytic (fibrin-splitting) action of plasmin, an enzyme that catalyzes the removal of fibrin. Plasmin is derived from plasminogen, an inert protein precursor that can be activated by tissue plasminogen activator. Streptokinase, urokinase, and tissue plasminogen activator are drugs that activate plasminogen and lead to the dissolution of clots (Waisman DM, Plasminogen: Structure, Activation and Regulation, 2003)

2.1.5 Synthesis of blood-clotting proteins

Almost every clotting protein is synthesized in the liver except FVIII which is additionally produced in a large number of other tissues (Wion et 1985). FIX, FX, prothrombin, FVII, protein C and protein S are synthesized in a precursor form and need vitamin K to complete their synthesis.

Table 1: Concentrations of coagulation factors required for normal hemostasis. With the exception of fibrinogen, factor levels are usually reported as percentages of the concentrations present in plasma pooled from normal individuals. Tissue factor is not present in plasma and cannot be quantified in patients.
Source: Tollefsen DM, Washington University in St. Louis:
http://tollefsen.wustl.edu/projects/coagulation/coagulation.html

Factor	Molecular Weight	Plasma Concentration (µg/ml)	Required for Hemostasis (% of normal concentration)
Fibrinogen	330,000	3000	30
Prothrombin	72,000	100	40
Factor V	300,000	10	10-15
Factor VII	50,000	0.5	5-10
Factor VIII	300,000	0.1	10-40
Factor IX	56,000	5	10-40
Factor X	56,000	10	10-15
Factor XI	160,000	5	20-30
Factor XIII	320,000	30	1-5
Factor XII	76,000	30	0
Prekallikrein	82,000	40	0
HMWK	108,000	100	0

2.2 Factor VIII (FVIII)

It was proven in 1840 that a bleeding disorder inherited within families, known as hemophilia, is due to a factor in the blood by correction of the bleeding defect by transfusion of whole blood. Decades later in 1937 a factor was shown to be effective in accelerating the coagulation process: antihemophilic globulin, today better known as factor VIII (FVIII).

FVIII is a glycoprotein, which is synthesized and released into the circulation by the liver. Additionally FVIII mRNA was found in spleen and other tissues but they are not yet completely identified (Jacquemin et al; 2006). After synthesis FVIII moves to the lumen of endoplasmatic

rediculum and is bound to proteins that regulate secretion. At the endoplasmatic rediculum cleavage of the signal peptide and addition of oligosaccharides occur. The chaperon proteins calinexin and calreticulin enhance secretion and degradation.

The primary translational product of FVIII is inefficiently transported from the endoplasmic reticulum to the Golgi apparatus (Dorner et al 1987), where several changes take place. For instance the posttranslational addition of sulphates to tyrosine residues that have a major impact on the procoagulant activity of FVIII and its interaction with von Willebrand Factor.

The FVIII gene was first discovered in 1984. It is located on the X-chromosome (Gitschier et al 1984), has a size of 186 kDa and is divided into 26 exons (Hoyer, 1994; Bi et al, 1995, Thompson 2003). The factor VIII protein consists of 2332 amino acids, arranged in this domain order A1-A2-B-A3-C1-C2 (Thompson 2003). Crystal structures of activated FVIII have been recently solved at just under 4 Angstrom resolution (Ngo et al 2008, Shen et al 2008).

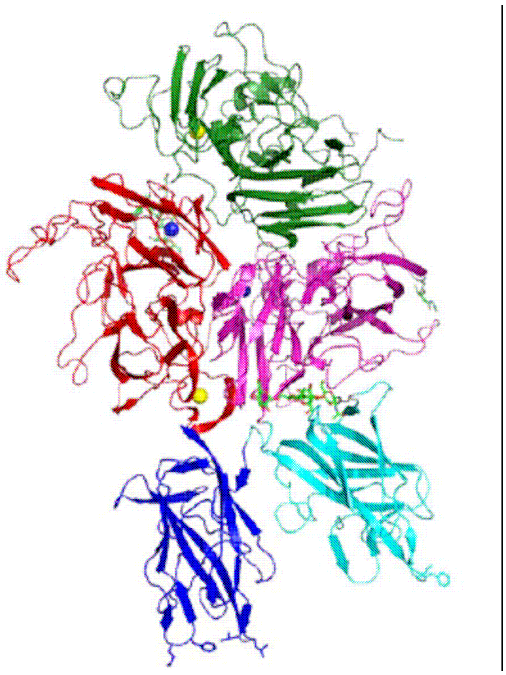

Figure 2: Crystal structure of FVIII: Shen et al 2008: The tertiary structure and domain organization of coagulation factor VIII. Blood 2008 Feb 1; 111(3):1240-7

Before the release into the circulation, the protein is proteolytically cleaved into a heavy chain, consisting of A1-A2-B domain and a light chain, consisting of A3-C1-C2 domain. (Vehar et al, 1984) The light chain contains the binding site for von Willebrand factor. Both parts of the heterodimer are kept together via a divalent Ca^{2+} ion.

Upon activation by thrombin (Factor IIa), FVIII dissociates from the complex with VWF to interact with Factor IXa and phospholipids. It is a cofactor to Factor IXa in the activation of Factor X to Factor Xa, which, in turn activates more thrombin together with its cofactor Factor Va. Thrombin cleaves fibrinogen into fibrin which polymerizes and cross-links (using Factor XIII) into a blood clot. No longer protected by VWF, activated FVIII is proteolytically inactivated in the process and quickly cleared from the blood stream (Colman et al, Hemostasis and Thrombosis 2001).

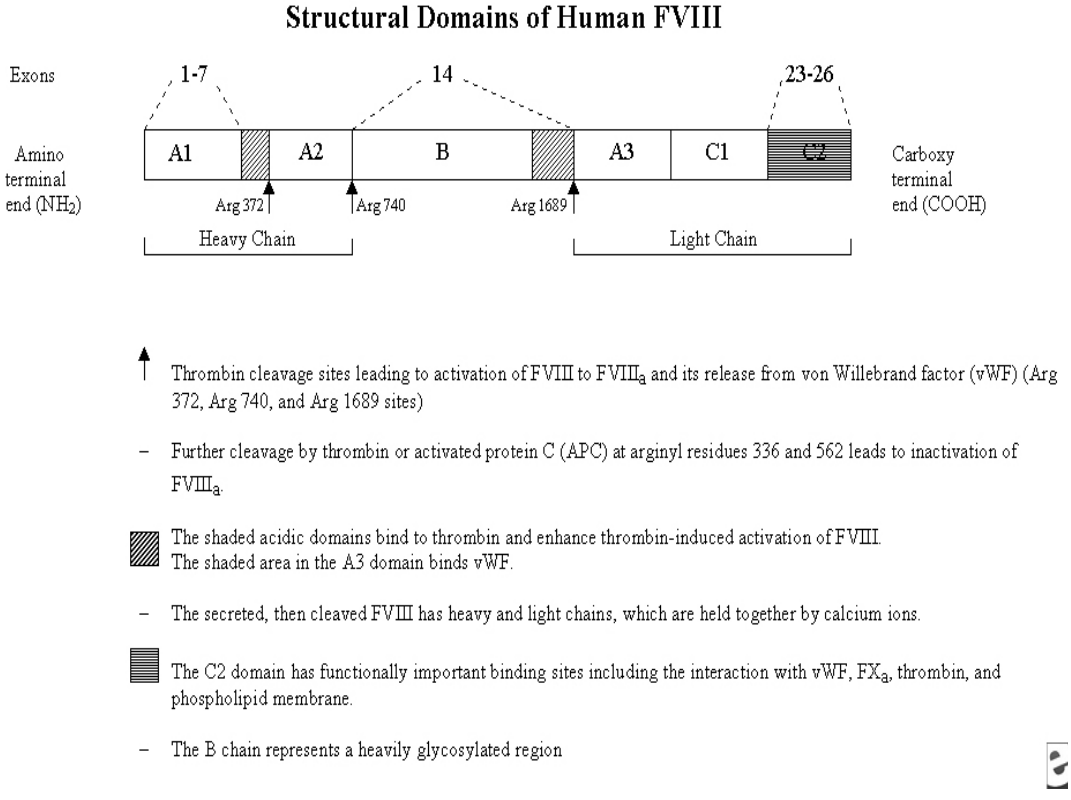

Figure 3: Factor VIII. Structural domains of human factor VIII. Adapted from Stoilova-McPhie S, Villoutreix BO, Mertens K, et al. 3-Dimensional structure of membrane-bound coagulation factor VIII: modeling of the factor VIII heterodimer within a 3-dimensional density map derived by electron crystallography. Blood 2002 Feb 15; 99(4): 1215-23. Roberts HR, Hoffman M. Hemophilia A and B. In: Beutler E, Lichtman MA, Coller BS, et al, eds. Williams Hematology. 6th ed. NY: McGraw-Hill; 2001:1639-1657. Roberts HR. Thoughts on the mechanism of action of F VIIa. Presented at: 2nd symposium on New Aspects of Haemophilia Treatment. 1991; Copenhagen, Denmark.

2.3 Hemophilia A

Hemophilia A is a bleeding disorder that is characterized by a deficiency in factor VIII clotting activity that is due to the lack or an insufficient amount of the clotting protein FVIII in the blood circulation. The cause of the disease is a genetic defect of the X-chromosome, which can be either inherited (or a spontaneous mutation. 80% of all Hemophilia patients are suffering from a FVIII deficiency.

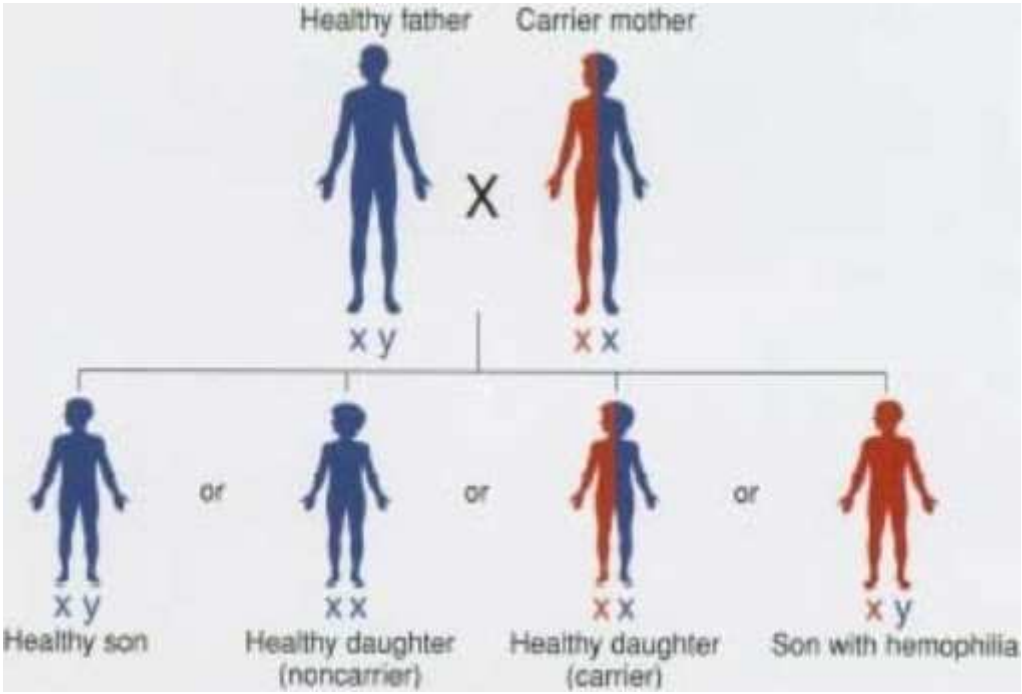

Figure 4: The gene for hemophilia is passed from mothers (XX) to sons (XY) via the X chromosome. A son who inherits a defective X chromosome from his mother does not have a healthy X chromosome to rely on the way daughters (XX) do.
Source: http://www.humanillnesses.com/original/Gas-Hep/Hemophilia.html

Due to the fact that the FVIII gene is located on the X-chromosome and is a recessive hereditary disease, it affects mainly male individuals. It's affecting about 1 of 5000-10000 men (Aledort et al, Textbook of Hemophilia 2005).

2.3.1 History of hemophilia A

The first recognition of excessive and unexplained bleeding was made in the Talmud, which is a collection of Jewish Rabbinical writings from the 2nd century AD (Talmud, Tractate Yevamot 64b). It was described that male babies did not have to be circumcised if two brothers already died from that procedure.

The Arabian physician Albucasis (12th century AD) wrote of males in a particular village, who had died of uncontrollable bleeding (Skinner 2001). The word “hemophilia”, from the Greek *haima* “blood” and philia “to love” (Harper D, Online Etymology Dictionary), first appeared in 1828 at the University of Zurich in a description of a bleeding disorder condition (Canadian Hemophilia Society, History of Hemophilia).

One of the most famous carriers of Hemophilia A was Queen Victoria of England (1837-1901) who passed the disease on to several royal families in Europe, including the Spanish, German and Russian Royals. Because of this, Hemophilia has often been called the “Royal disease” (Potts 1999)

2.3.2 Symptoms and diagnosis of hemophilia A

People with untreated hemophilia A are suffering from prolonged bleeding after injuries, tooth extractions or surgery, or having renewed bleeding after the initial bleeding already has stopped(Kessler & Mariani 2006). Muscle hematomas or inter-cranial bleeding can occur even 4 to 5 days after the injury. Other typical symptom for hemophilia A patients are spontaneous bleeding events, especially in the joints like knees, ankles and elbows.

The time of diagnosis and the frequency of the bleeding episodes depend mainly on the severity of disease. However, the bleeding events are more frequent in childhood and adolescence than in adulthood. This could be due to physical activity levels as well as vulnerability during more rapid growth.

3 severities of hemophilia A are:

Severe hemophilia A:

We are talking of severe hemophilia A when the FVIII clotting activity is below 1% of normal. The diagnosis for people with severe hemophilia A happens most of the time during their first year of life. Sometimes they already have extra- or intracranial bleedings following birth. In some babies, hemophilia is suspected immediately when a routine circumcision results in unusually heavy bleeding. Small children are at particular risk because they frequently fall and may bleed into the soft tissue of their arms and legs. These small bleeds result in bruising and noticeable lumps but do not usually require treatment. As a child becomes more active, bleeding may occur into the muscles, a much more painful and debilitating situation. These muscle bleeds result in pain and pressure on the nerves in the area of the bleed. Damage to nerves can cause numbness and decreased ability to use the injured limb. Patients do have in average 2-5 spontaneous bleeding events per month. Though the joints are the common site where bleeding occurs, it happens on other sites as well like in kidneys, gastrointestinal tract and brain. Without treatment patients suffer from prolonged bleeding episodes or excessive pain and swelling from minor injuries (Aledort et al, Textbook of Hemophilia 2005).

Moderately severe hemophilia A:

Patients with moderately severe hemophilia A do have a FVIII clotting activity between 1 and 5 % of normal. With this level of functional FVIII, spontaneous bleeding is rare, but a prolonged bleeding after a relatively minor trauma is common. Usually it is diagnosed before the age of 5 or 6. Bleeding episodes occur between once a month to once a year. The signs and the symptoms for bleeding are the same as for severe hemophilia A (Aledort et al, Textbook of Hemophilia 2005).

Mild hemophilia A

Individuals with mild hemophilia A do have a FVIII clotting activity between 6 and 35% of normal. Abnormal bleeding occurs with surgery, tooth

extractions and major injuries. Patients never have spontaneous bleedings. Therefore, they are often not diagnosed until later in life when they have for example a surgery or a major trauma. Bleeding episodes occur between once a year and once every ten years (Aledort et al, Textbook of Hemophilia 2005).

Although female carriers of hemophilia A do not have the disease, they could have a FVIII clotting activity below 35% which could lead to symptoms like in male patients with mild hemophilia A. Moreover it was shown, that female carriers with FVIII activity between 35% and 60% may have subtle abnormal bleeding (Plug et al 2006).

Table 2*:* Symptoms related to severity of untreated hemophilia A. Adopted from: Aledort et al, Textbook of Hemophilia 2005

Severity	**Factor VIII clotting activity**	**Symptoms**	**Usual Age of Diagnosis**
Severe	<1%	Frequent spontaneous bleeding; abnormal bleeding	1st year of life
Moderately severe	1%-5%	Spontaneous bleeding is rare; abnormal bleeding after *minor* injuries, surgery, or tooth extractions	Before age 5-6 years
Mild	>6%-35%	No spontaneous bleeding; abnormal bleeding after *major* injuries, surgery, or tooth extractions	Often later in life

2.3.3 Molecular genetics of hemophilia A

There are several pathologic allelic variants of the factor VIII gene that lead to hemophilia A. First of all a gene inversion through recombination between a sequence located within intron 22 with one end of the two additional copies of homologues sequence near the telomere of the long arm of the X-chromosome (Bagnall et al 2006). This mutation is the cause for about 45% of all severe hemophilia A cases (Kaufman et al 2006).

A different recurrent inversion occurs between a 1-kb sequence in intron 1 that is repeated in the reverse orientation to the factor VIII gene (Bagnall et al 2002); this type occurs in up to 3% of families with severe hemophilia A. The rest of the mutations cover the entire spectrum: a complete or partial deletion of FVIII, large insertions, sequence duplications, frame shifts, splice junction alterations, nonsense mutations and missense mutations. The resulting gene product goes from total absence of detectable protein (majority of individuals with severe hemophilia A) to those with normal levels of a dysfunctional protein (Aledort et al, Textbook of Hemophilia 2005).

2.3.4 Treatment of hemophilia A patients

Before 1960, patients with severe hemophilia A had a life expectancy of only 25 years. But this has been changed over the last four decades through advancements in treatment that permitted a near-normal lifestyle and life span for many individuals with hemophilia (Darby et al 2007). Nowadays hemophilia A patients in the western world are treated by administering a replacement therapy with FVIII concentrates which could either be plasma-derived or recombinant. In the 1970s the introduction of factor VIII concentrates revolutionized the therapy. It allowed treatment at home and largely replaced the need for transfusion of whole blood or plasma in the hospital (Pool et al 1964). But the other side of the coin was infection with hepatitis and HIV from products made from pooled plasma. In the early 1980s about 70% of hemophiliac patients were infected with HIV. Therefore, a virucidal treatment of plasma-derived FVIII concentrates has been established, which lower the risk of HIV transmission since 1985 and for hepatitis B and C since 1990. Moreover a recombinant FVIII concentrate was developed, which today provide no content of any human or animal derived proteins (Aledort et al, Textbook of Hemophilia 2005).

Factor VIII is given by direct injection either on demand or prophylactic. On demand treatment is just for individuals with mild or moderately severe hemophilia. FVIII is administered when bleeding occurs in order to stop the bleeding as soon as possible and prevent further damage from bleeding.

People with severe hemophilia are receiving a prophylactic treatment. It is a regular continuous treatment with FVIII concentrate to keep the level of the clotting factor in the blood high enough to prevent spontaneous bleeding and decrease the number of bleeding episodes. Prophylactic infusion almost completely eliminates joint bleeding and therefore greatly decrease chronic joint disease. The benefit of prophylactic treatment for people with severe hemophilia is not questioned, but there is still a controversy when to start with. The age when a child first experiences a joint bleeding can vary greatly. Moreover a prophylactic treatment is very expensive and only patients in the western world can afford the costs of a treatment (Aledort et al, Textbook of Hemophilia 2005).

Although most individuals with hemophilia can use replacement products repeatedly without problems, one of the most problematic complications of treatment is the development of inhibitors to FVIII (Ehrenforth et al 1992 ; Hoyer et al 1995). Inhibitors are typically IgG antibodies against FVIII that neutralize the coagulant effects of replacement therapy. Inhibitors to FVIII occur approximately in 20-30% of severe hemophilia A patients. Management for patients who developed inhibitors includes the eradication of circulating FVIII neutralizing antibody via immune tolerance induction (ITI). The first reports on immune tolerance induction as treatment of hemophiliacs, were proposed by Brackmann and Gromsen in 1977 (Brackman et al 1977). This procedure is only effective in 60-80% of the patients (DiMichele 2000). The other patients have to take bypassing products such as Feiba or FVIIa.

2.4 Extension of factor VIII half-life

For patients with prophylactic treatment it is necessary to receive about 3 times a week FVIII concentrate injections to keep the level of FVIII in blood high enough to prevent spontaneous bleeding. This is due to the half-life of an average of 12 hours of FVIII in circulating blood. Baxter Innovations GmbH is currently trying to improve this procedure by elongating the half-life of FVIII to make the therapy more convenient for the patients.

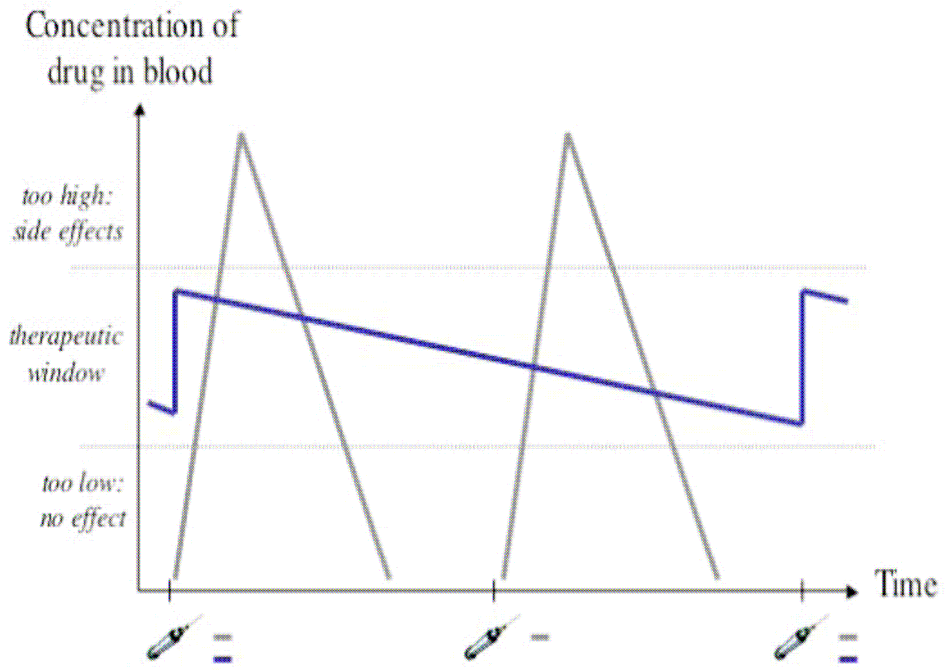

Figure 5: The idealized pharmacokinetic profile of an unmodified drug compound (grey) and its optimized second-generation version (blue): Rottensteiner HP, Baxter BioScience, unpublished

Injection of an unmodified drug lead to the need of more injections. Moreover you end up with too high concentration in the blood at the time of injection and too low concentration after a relative quick period of time. In theory an engineered compound requires fewer injections and provides for a better match within he therapeutic window (Figure 5).

Baxter Innovations GmbH is trying to elongate the half-life of FVIII by chemical modification with polyethylene glycol (PEG) of rFVIII itself using ADVATE or rVWF, which serves as the carrier molecule of FVIII.

2.5 Polyethylene glycol (PEG)

Many biopharmaceuticals especially proteins and peptides have typically problems like a short circulating half-life, immunogenicity, proteolytic degradation and low solubility. There are several ways to come over this problem. For instance manipulation of the amino acid sequence to decrease immunogenicity and proteolytic cleavage, fusion or conjugation to immunglobulins and serum proteins, incorporation into drug delivery vehicles for protection and slow release, and conjugation to natural or synthetic polymers (MJ Roberts et al 2002).

Polyethylene glycol (PEG) is a highly investigated polymer for the covalent modification of biological macromolecules and surfaces for many pharmaceutical and biotechnical applications. In the last years the scientific community has become quite familiar with the improved pharmacological and biological properties that are associated with the covalent attachment of PEG (PEGylation) to therapeutically useful polypeptides (MJ Roberts et al 2002).

For example, PEGylation can shield antigenic epitopes of the polypeptide and therefore reduce reticuloendothelial (RES) clearance and recognition by the immune system. During the last years it has been realized that the chemistry and the quality of the used PEG reagents play an important role for peptide and protein modification. Therefore a second generation of PEG chemistry has been established to avoid the problems that are associated with PEG-protein products that were developed using the first generation of PEG chemistry. Those problems were deactivated PEG impurities, restriction to low molecular weight mPEG, unstable linkages and lack of selectivity in modification (MJ Roberts et al 2002).

2.5.1 Properties of polyethylene glycol

In its most common form polyethylene glycol is a linear or branched polyether terminated with hydroxyl groups and having the general structure:

$$HO - (CH_2\text{-}CH_2\text{-}O)_n - CH_2\ CH_2 - OH$$

PEG is synthesized by anionic ring opening polymerization of ethylene oxide starting with a nucleophilic attack of a hydroxide ion on the expoxide ring. An important aspect of PEGylation is the incorporation of various PEG functional groups that are used to attach the PEG to the peptide or protein. Most useful for polypeptide modification is monomethoxy PEG, mPEG, having the general structure:

$$CH_3O - (CH_2\text{-}CH_2\text{-}O)_n - CH_2\ CH_2 - OH$$

Compared with other polymers, PEG has a relatively narrow polydispersity in the range of 1.01 for low molecular weight PEGs (<5kDa) to 1.1 for high molecular weight PEGs (>50kDa). It is calculated by dividing the weight average molecular weight with the number average molecular weight. Polydispersity indicates the distribution of individual molecular masses in a batch of polymers (MJ Roberts et al 2002).

PEG is soluble in both aqueous and organic solutions, which makes it suitable for end group derivatization and chemical conjugation to biological molecules under mild physiological conditions.

Another important property of PEG is the binding of 2-3 water molecules per ethylene in solution. Therefore and because of the high flexibility of the backbone chain, PEG acts if it were 5-10 times as large as a soluble protein of comparable molecular weight. These factors have been suggested as the reason that PEG exhibits the ability to precipitate proteins (Polson 1977), exclude proteins and cells from surfaces (Gombotz 1992), reduce immunogenicity and antigenicity (Abuchowski

1977) and prevent degradation by mammalian cells and enzymes (Working 1997).

PEG clearance occurs without structural change and depends on the molecular weight. PEGs with a size below 20kDa get cleared through urine, PEGs higher than 20kDa get cleared more slowly in the urine and feces (MJ Roberts et al 2002).

It has been shown that low molecular weight oligomers of PEG (<400Da) degraded in vivo to toxic metabolites, but lack of toxicity with a molecular weight above 1000 Da has been revealed over many years of use in foods, cosmetics and pharmaceuticals (Working 1997).

2.5.2 Chemistry of PEGylation

For the coupling reaction between the PEG and the molecule of interest, it is necessary to activate PEG by making a derivative of the PEG having a functional group at one or both termini. The choice which functional group to take is depending on the reactive groups on the molecule that will be coupled.

$$HO - (CH_2\text{-}CH_2\text{-}O)_n - X \quad \text{or} \quad MeO - (CH_2\text{-}CH_2\text{-}O)_n - X$$

X: functional group for coupling to protein

The most common route for PEG conjugation of proteins has been to activate the PEG with functional groups suitable for reactions with lysine and N-terminal amino acid groups.

The monofunctionality of methoxyPEG makes it particularly suitable for protein and peptide modification because it yields reactive PEGs that do not produce cross-linked polypeptides, as long as diol PEG has been removed (MJ Roberts et al 2002).

2.5.3 PEG structures

Branched structures of the PEG have been proven to be very useful in addition to the linear structures when you PEGylate a protein or a peptide. For example a branched PEG attached to a protein acts much larger than a corresponding linear mPEG of the same molecular weight (Yamasaki 1988). This structure of PEG also has the advantage of adding two PEG chains per attachment site on the protein, therefore reducing the chance of protein inactivation. Furthermore, this structure is more effective in protecting proteins from proteolysis, reducing antigenicity and reducing immunogenicity (Veronese 1997).

2.5.4 PEGylation of FVIII

Baxter Innovations GmbH has chosen to do the PEGylation with a branched stable PEG reagent to chemically modify FVIII in order to increase its half-life in circulating blood.

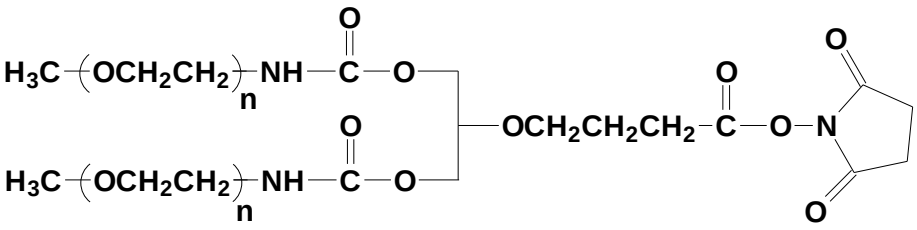

Figure 6: Structure of Nektars stable PEG reagent: PEG2ruNHS 20K. Nektar Therapeutics, San Carlos USA

The structure of the stable PEGylation reagent (PEG2ruNHS20K) is shown in Figure 6. The two PEG chains are connected to a glycerol backbone via a reversed urethane segment. In addition an active N-hydroxsuccinimide (NHS) ester is linked to the glycerol residue in position 2 via a propanoxy group. The NHS ester can react with primary amino groups (N-terminus and ε-amino groups of lysines) to form a stable amide bond.

In Figure 7 the structure of the conjugate after PEGylation with the stable PEGylation reagent is shown.

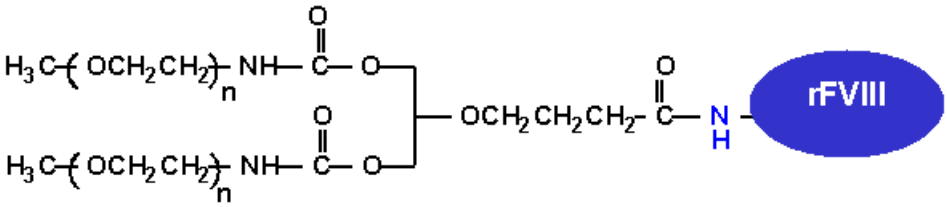

Figure 7: Structure of the conjugate. Siekmann J, Baxter BioScience, unpublished

2.6 Anti-PEG antibodies

To evaluate the incidence of anti-PEG antibodies within a normal, healthy human population, Armstrong et al analyzed 250 plasma samples of normal donors (Armstrong JK et al, ASH 2003) employing the methods as described by Fisher et al. 25.2% of samples were positive for IgG and/or IgM, of which 18.4% showed IgG binding only, 3.6% IgM only, and 3.2% both. A further study performed in ALL subjects receiving PEG-Asparaginase demonstrated the development of anti-PEG antibodies (Armstrong JK et al, ASH 2006): 9 of 15 subjects who showed undetectable asparaginase activity tested positive by serology and 13 when using the flow cytometry, whereas a cohort of 13 subjects with normal sustained levels of asparaginase activity after treatment did not develop anti-PEG antibodies. In approximately one third of the patients having developed anti-PEG antibodies, rapid clearance of PEG-Asparaginase rendered treatment ineffective. No relationship was observed between the presence of anti-PEG antibodies and serum asparaginase activity for patients treated with unmodified asparaginase.

These findings of anti-PEG antibodies raise the question whether

1. the presence of anti-PEG antibodies may alter the pharmacokinetic profile of the pegylated conjugate and eventually render treatment less effective or ineffective, necessitating a modification of dosing recommendations;
2. pegylated coagulation factors induce anti-PEG antibodies leading to a less effective treatment as mentioned in 1).

2.7 Objectives of the diploma project

The aim of my project was to establish an anti-PEG antibody detection assay. The assay had to be stable, reproducible, reliable, accurate and specific for anti-PEG antibodies in order to comply with quality requirements. Once the test was established it was used to monitor the development of anti-PEG antibodies in disease models in mouse and rat during preclinical studies of new drug candidates that are currently developed by Baxter Innovations GmbH as well as for screening healthy human blood donors for the potential presence of “natural” anti-PEG antibodies. This study in healthy blood donors provides important information for the screening of patients to be included in clinical studies of the new PEGylated protein drug candidates.

3. Material and Methods

3.1 Material

3.1.1 Reagents

Tentagel M NH2	Rapp Polymere Thübingen, Germany
D-PBS	Invitrogen Carlsbad, USA
Anti-PEG monoclonal IgM (Clone Number: AGP3)	Academia Sinica Nanking, Taiwan
Anti-PEG monoclonal IgG1 (Clone Number: E11)	Academia Sinica Nanking, Taiwan
Anti-PEG rabbit monoclonal IgG (Clone ID:PEG-B-47)	Epitomics Burlingame, USA
Anti-PEG/Albumin from rabbit, protein A purified	Baxter BioScience Deerfield, USA
Goat anti-mouse IgM-RPE, Human adsorbed	Southern Biotech Birmingham, USA
Goat anti-mouse IgG1	Southern Biotech Birmingham, USA
Goat anti-rat IgG-RPE	Southern Biotech Birmingham, USA
PEG2ruNHS 20K	Nektar Therapeutics San Carlos, USA

HEPES	Merck Darmstadt, Germany
NaCl	Merck Darmstadt, Germany
HCl	Merck Darmstadt, Germany
Glycine	Merck Darmstadt, Germany
WFI	Baxter BioScience Deerfield, USA
APC Conjugation KIT	AbD Serotec Oxford, UK

3.1.2 Plastic & Consumables

Pipette tips (20, 200, 1000µl)	Eppendorf Hamburg, Germany
Combitips	Eppendorf Hamburg, Germany
Tubes 30ml	Sterilin Hounslow, UK
Polypropylene tubes 1,4ml	Micronic Lelystad, Netherlands
Polysterene round bottom, 5ml	Becton Dickinson Franklin Lakes, USA

CellTrics disposable filter, 30µm	Partec Görlitz, Germany

3.1.3 Buffers

Buffer A	50mM HEPES 100mM NaCl WFI pH: 7,6
Buffer B	20mM HEPES 150mM NaCl WFI pH: 7,4
2mM HCl	
01M Glycin solution	

3.1.4 Equipment

Pipettes (0,5-10µl, 10-100µl, 100-1000µl)	Eppendorf Hamburg, Germany
Multipipette	Eppendorf Hamburg, Germany
Stuart Vortex Mixer	SciLabware Limited Staffordshire, UK
Lab-scale	Sartorius Göttingen, Germany
FACS Calibur	Becton Dickinson Franklin Lakes, USA

Heraeus Centrifuge	DJB Labcare Ltd Buckinghamshire, UK
Microcentrifuge	Eppendorf Hamburg, Germany
Ultrasonic bath	Elma Singen, Germany
Particle counter	Beckman Coulter Fullerton, USA
Boxer Gas pump	Uno International Ltd. London, UK

3.2 Methods

3.2.1 Flow Cytometry

Flow Cytometry is a powerful technique for the analyses of multiple parameters of individual cells within heterogeneous populations. This technique can be used for many applications, for instance immunophenotyping, ploidy analyses, cell counting or GFP expression analyses. The basic principle of this approach is the emerging light after cells pass a laser beam that can be gathered and give rise about cell characteristics such as size, complexity, phenotype and health (Langobardi Givan 2001).

Instrument Overview

- Fluidic system: presents the sample to the interrogation point and takes away the waste. For accurate data collection the particles or cells have to pass the laser beam one by one. For this purpose the sample stream is injected into a flowing stream of sheath fluid or saline solution. The sample stream gets compressed to one cell in diameter. This process is called hydrodynamic focusing.
- Laser: The light source for scatter and fluorescence
- Optics: gathering and direct the light
- Detector: receive the light
- Electronics and peripheral computer system: convert signals from the detectors into digital data and do the analyses.

The interrogation point is the heart of the system. It is the point where the laser and the sample intersect and where the optics collect the resulting scatter and fluorescence.

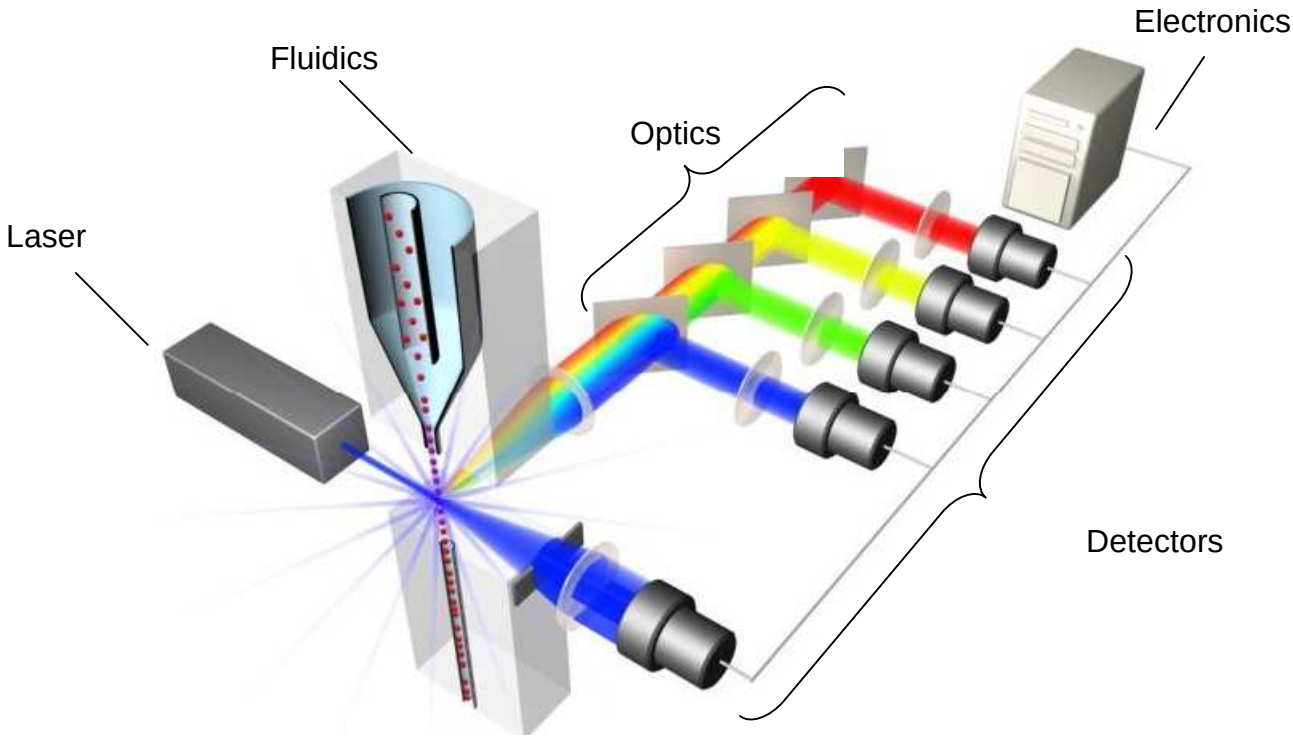

Figure 8: Overview of a flow cytometric system. Invitrogen: Tutorials on fluorescence and flow cytometry. http://www.invitrogen.com/site/us/en/home/support/Tutorials.reg.at.html

When a cell pass the laser beam it scatters light at all angles.

Forward Scatter

The forward scatter is the amount of light that is scattered in the forward direction as laser strikes the cell. The scattered light is collected by a detector that converts intensity into a voltage pulse. The magnitude of this pulse is proportional to the size of the cell and can therefore be used to quantify that parameter. Small cells produce a small amount of forward scatter, large cells a large amount. An obscuration bar is placed in front of the detector to prevent any of the intense laser light reaching the detector. When plotting the data on a histogram, smaller cells appear left, bigger cells right (Langobardi Givan 2001).

Side Scatter

The light scattering at larger angles, for example to the side, is caused by granularity and structural complexity inside the cell. Side scattered light is focused through a lens system and collected by a detector, usually located

90 degree from the laser's path. Recording of the data is the same as for forward scatter (Langobardi Givan 2001).

Fluorescence

The term fluorescence is used to describe the excitation of a fluorophore to a higher energy level followed be the return of that fluorophore to the ground state with the emission of light.

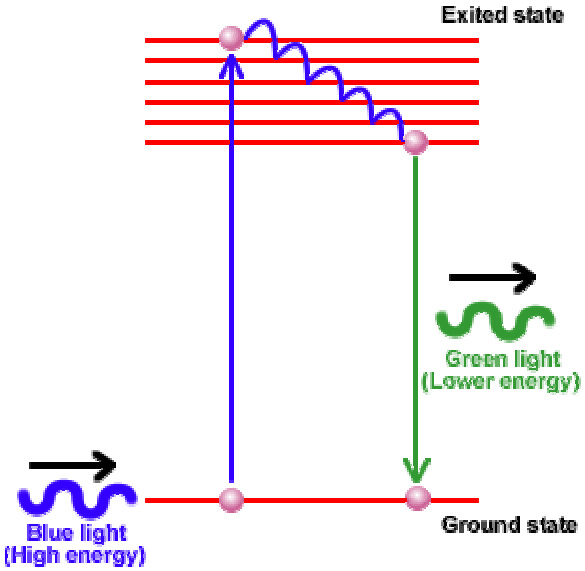

Figure 9: Principle of fluorescence: a fluorophore that can absorb blue light. Because the photonic energy is related to the wavelength there is a difference in the colour light that is absorbed to that which is emitted.
Source: Centre for synaptic plasticity, University of Bristol.
http://www.bristol.ac.uk/synaptic/info/imaging/imaging_1.htm

One of the major applications in flow cytometry is to study cellular characteristics by fluorophore-labeled antibodies that bind to cell specific proteins. When a cell passes through the laser beam with the right wavelength, the fluorophore gets excited and emits light at a specific wavelength that gets collected by a detector. The fluorescent light coming from labeled cells as they pass the laser travels along the same way as the side scatter signal. After passing a series of filters and mirrors, the light with the right wavelength is delivered to the appropriate detectors. There the signal is translated to a voltage pulse that is proportional to the amount of fluorescence emitted, similar to forward- and side scatter (Langobardi Givan 2001).

Gating

One of the major analyses tools in flow cytometry is gating. This tool enables us to direct the analyses software to consider only specific cells or particles for further analyses (Langobardi Givan 2001).

3.2.2 Chemical modification of TentaGel M NH2 with a branched stable PEG reagent

TentaGel M NH2 Beads (capacity: 0.24 mmol/g) were provided by Rapp Polymere. The primary amino group of the PEG chain was chemically modified by use of a branched PEGylation reagent PEG2ruNHS 20K, containing an active N-hydroxysuccinimide (NHS) group. 450 mg TentaGel M NH2 Beads were suspended in 20 ml Buffer A. Then the suspension was centrifuged for 30 min (2800 RCF). The supernatant was discarded and the remaining beads were suspended again in 20 ml Buffer A. The suspension was gently shaken for five minutes and placed in an ultrasonic bath for 30 sec to avoid aggregates. Then the washing procedure was repeated. Finally the pH of the suspension was controlled. Then the PEGylation reagent was dissolved in 2 mM HCl (75 mg/ml) and added to the beads suspension to give a final concentration of 0.6 mg PEG reagent/mg TentaGel. The PEGylation reaction was performed for 2 h at room temperature under gentle shaking. Subsequently the reaction was terminated by addition of a 0.1M Glycine solution (final concentration: 0.01M). This solution was gently shaken again for 1 h at room temperature. Then the PEGylated beads were centrifuged and resuspended with Buffer B as described above. This washing procedure was performed three times. Finally the beads suspension was resuspended in Buffer B and lyophilized.

3.2.3 Anti-PEG antibody detection assay

Bead preparation

Anti- polyethylene glycol (PEG) antibodies were detected by TentaGel M NH2 microspheres. Those polystyrene beads (diameter: 10 µM) are grafted with PEG and were additionally conjugated with branched PEG

reagent. After conjugation reaction the beads were lyophilized. and stored at 4℃.

Before using the beads they had to get reconstituted in PBS and left for at least 1 hour to have time for swelling. The beads were washed with 20ml PBS, centrifuged at 300g for 5 minutes and the supernatant was discarded. This step was one time repeated and the pellet was reconstituted with an appropriate amount of PBS to get a 1% solution. Short sonification and filtration of the bead-solution should prevent any formation of clumps. The beads were counted and diluted if required to get a final concentration of about $2x10^6$/ml.

Sample preparation and anti-PEG antibody detection reaction

The blood samples taken from humans or animals were centrifuged for 10 minutes at 1100g and once more for 20 minutes in order to isolate the plasma. Supernatant was collected and was ready for further use in the detection assay.

We filled 25 µl of the bead solution together with 25µl of the plasma samples into micronic tubes and vortex them. Incubation step occurred at room temperature for 30 minutes. The reaction was stopped by filling 1ml PBS to the solution. Now we centrifuged at 200g for 2 minutes and evacuated the supernatant till 50µl are left in the tube. This washing step was repeated once more.

To detect antibodies that were bound to the surface of the Tentagel beads we took secondary antibodies labeled with fluorochromes that were directed against those primary antibodies. The secondary antibodies were diluted 1:32 with PBS and 5µl of each antibody was given to the micronic tubes. The tubes were vortexed and the incubation was running for 30 minutes at room temperature in the dark. The reaction was stopped again by filling 1ml PBS to the solution. Now we centrifuged at 200g for 2 minutes and evacuated the supernatant till 50µl are left in the tube. This washing step was repeated once more and the pellet was reconstituted in 50µl PBS.

The samples were now analyzed by flow cytometry.

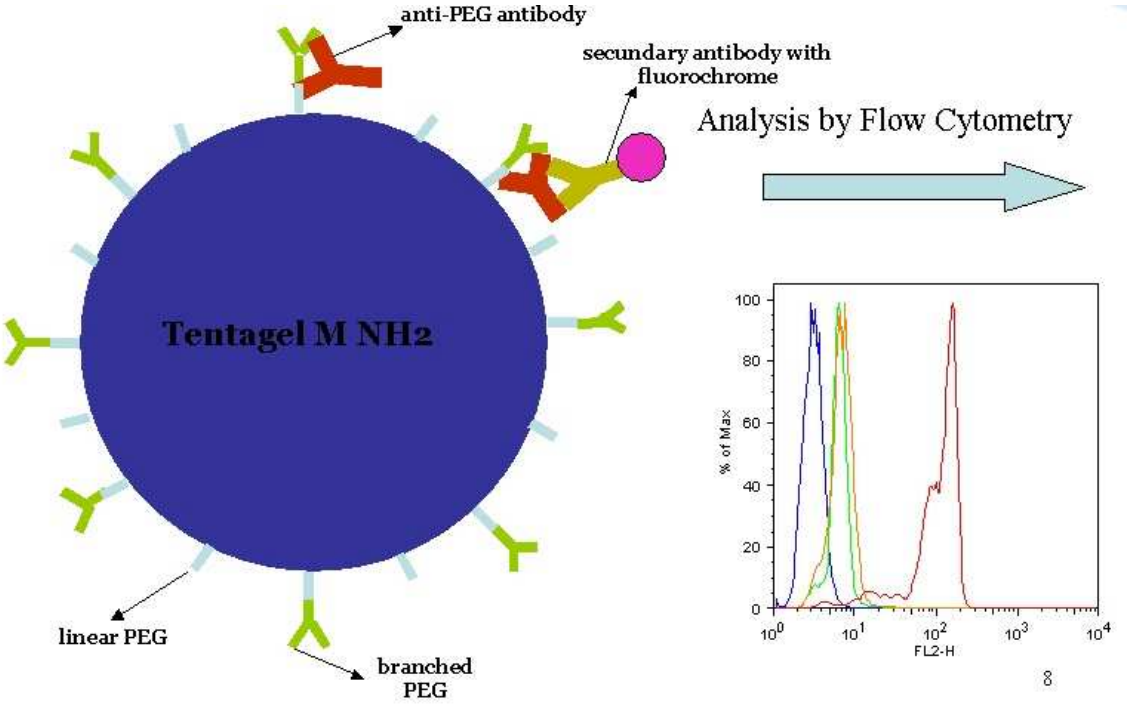

Figure 10 *:* Overview of the anti-PEG antibody detection assay

All experiments that were done to detect anti-PEG antibodies were done with this procedure if not otherwise indicated.

3.2.4 Experimental set ups to establish the anti-PEG antibody detection assay

Establishing the assay

The first experiments were done using a protein A purified IgG antibody from rabbits that were treated before with PEG-FVIII. TentaGel Beads from Rapp polymere without additional PEGylation were used in this experiment. As negative control unstained beads and beads incubated with the secondary antibody were used.

Titration of the beads

To see whether the concentration of the beads has an influence on the detection of the anti-PEG antibodies, a titration of the beads was done.

The bead solution was serially diluted with PBS and different dilutions were tested using the rabbit anti-PEG antibody in 1:2 dilution with PBS as a positive control. As negative control unstained beads and beads incubated with the secondary antibody only were used.

Antibody titration

Commercially available monoclonal murine IgG and IgM anti-PEG antibodies were ordered to use them in future as a positive control to check for proper operation of the assay. A titration of each primary and secondary antibody was done to find out the best dilution. Lowering the concentration of the antibody to a point that would be necessary to see a positive signal, enabled us to exclude unspecific binding on the one hand, and on the other hand reduce the costs of each experiment. Each antibody was diluted with PBS and different concentrations were tested.

TentaGel vs. TentaGel with branched PEG

To see if the additional PEGylation of the TentaGel beads had an influence on the assay, a comparison between both bead types was done. For this purpose, we used mice plasma samples that had been tested positive for IgM anti-PEG antibodies before. The plasma samples were diluted with PBS 1:20 and were tested with both bead types.

Storage and stability tests

Another important issue was to ensure that the beads used for analyses are stable, first of all in terms of PEGylation, and how to store them best without destabilizing. To encounter that question, one lot of the beads was stored under 4℃ and tested several times during a time period of 4 weeks.

In addition three different batches of beads with additional PEGylation were produced and lyophilized. Every batch was stored under 3 different conditions, namely 4℃, room temperature and 37℃. Analyses were done shortly after production and after 4 weeks of storing.

A murine monoclonal IgM anti-PEG antibody was used as positive control, the unstained beads and the beads incubated with secondary antibody only were used as negative control.

Competition Assay

To see whether the test is specific for anti-PEG antibodies a competition assay was established. The positive control was divided in two aliquots: One aliquot was pre-incubated with 5µl of soluble PEG (wt 200) in different dilutions for 30 minutes at room temperature, the other aliquot was incubated with 5µl PBS. The dilutions of the soluble PEG were done geometrically with PBS. A monoclonal rabbit IgG anti-PEG antibody was used as a positive control. As negative control the unstained beads and the beads incubated with the secondary antibody were taken.

3.2.5 Cut off experiments in mouse

The aim of this experiment was to define the cut off in mice that was needed to distinguish between positive and negative plasma samples. For this purpose blood samples from 53 naïve mice, which means they had no treatment with polyethylene glycol, were taken and plasma analyzed with the anti-PEG antibody detection assay. The plasma samples were pre-diluted with PBS to have an end concentration of 1:20, 1:40, 1:80 and 1:160. The murine monoclonal antibodies against PEG were used as positive control, unstained beads and beads incubated with secondary antibody only were used as negative control.

The results obtained from the 1:20 dilution were taken to define the cut off by calculating the 95% percentile using the software program SPSS (SPSS Inc., Chicago, USA).

3.2.6 Statistical method to define the cut off: 95 % percentile

A percentile is the value of a variable below which a certain percent of observations falls. The 95th percentile is the value (or score) below which 95 percent of the observations are found. The term percentile and the

related term percentile rank are often used in descriptive statistics as well as in the reporting of scores from norm-referenced tests.

SPSS is using following method to estimate the value, v_p, of the p^{th} percentile of an ascending ordered dataset containing N elements with values $v_1, v_2, \ldots, v_N$;

$$n = \frac{p}{100}(N-1)+1$$

n is then split into its integer component, k and decimal component, d, such that $n = k + d$

If $N = 1$, the value for the percentile, v_p, is the only member of the dataset, v_1.

Else If $k = N$, then $v_p = v_{k-1} + d(v_k - v_{k-1})$.

Else $(1 < k < N)$ then $v_p = v_k + d(v_{k+1} - v_k)$.

(David Lane, “Percentiles” 2007)

3.2.7 Monitoring of potential anti-PEG antibody development in mice treated with PEGylated proteins

In the course of the factor VIII Half-life (FVIII-HL) project (extension of the factor VIII half-life) another important protein within the coagulation process was PEGylated: von Willebrand factor (VWF). VWF is the carrier molecule of FVIII. Several studies have been done to compare the immunogenicity of recombinant von Willebrand factor (rVWF) and PEGylated rVWF. In all experiments, humanized E17 mice were used.

Humanized E17 mice

MHC-class II molecules are not only crucial for the regulation of adaptive immune responses against protein antigens but are also essential for shaping the $CD4^+$ T-cell repertoire in the thymus (von Boehmer 2003). Structural differences between the murine and the human MHC-class II complex belong to the major limitations in the use of conventional murine disease models for dissecting immune responses to human protein antigens. In view of these limitations, considerable efforts have been put into the development of murine disease models that carry transgens for

human MHC-class II on the background of a knockout of the murine MHC-class II genes (Sonderstrup G 1999, Vandenbark AA 2003). However, appropriate models for hemophilia A were still missing. To overcome this limitation, Baxter Innovations GmbH developed a humanized murine model for hemophilia A in which the regulation of anti-FVIII immune responses is driven by FVIII-derived peptides that are presented by the human MHC-class II haplotype HLA-DRB1*1501 (Reipert et al 2007).

Comparative immunogenicity of rVWF and PEG-rVWF in humanized E17 mice: study 1

The aim of the study was to compare the immunogenicity of rVWF and PEG-rVWF. Mice were treated with different doses, in the presence or absence of the FVIII product ADVATE, which is Baxter's human recombinant FVIII (Table 3: Study design). All samples were tested twice for anti-PEG antibodies. Murine anti-PEG antibodies were used as positive control. Unstained beads and beads incubated with the secondary antibody only were taken as negative control.

Table 3: Study design of study 1: comparative immunogenicity of rVWF and PEG-rVWF in mice

Group	animal number	Target dose
1	10 male	FVIII only 1 µg in 200µl
2	10 male	VWF only 20 µg in 200 µl
3	10 male	PEG-VWF only 20 µg in 200 µl
4	10 male	VWF+FVIII 20 µg VWF + 1 µg FVIII in 200 µl
5	10 male	PEG-VWF+FVIII 20 µg PEG-VWF + 1 µg FVIII in 200 µl

The mice were treated after following schedule:

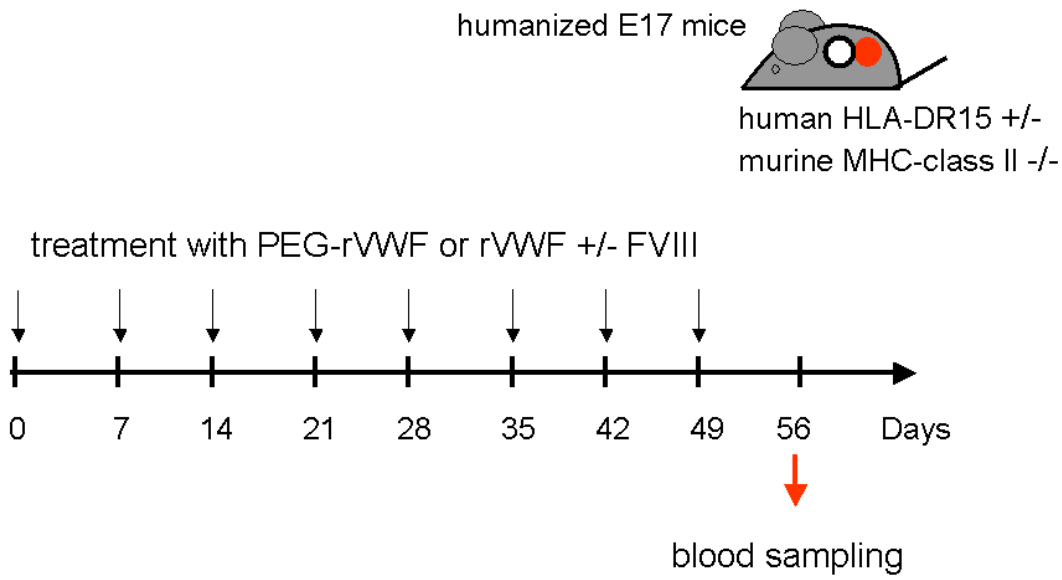

Figure 11: Treatment schedule for study 1: comparative immunogenicity of rVWF and PEG-rVWF in mice

Comparative immunogenicity of rVWF and PEG-rVWF in humanized E17 mice: study 2

The aim of the study was to compare the immunogenicity of rVWF and PEG-rVWF. Mice were treated with different doses, in the presence or absence of the FVIII product ADVATE, which is Baxter's human recombinant FVIII (Table 5). All samples were tested twice for anti-PEG antibodies. All samples were titrated with PBS starting with a 1:20 dilution to define the FACS titer endpoint. FACS titer endpoints were converted to FACS log 2 titer values:

Table 4: FACS titer endpoint conversion to FACS titer log 2 values

FACS Titer endpoint	FACS titer log 2
<1:20	0
1:20	1
1:40	2
1:80	3

Murine anti-PEG antibodies were used as positive control. Unstained beads and beads incubated with the secondary antibody only were taken as negative control.

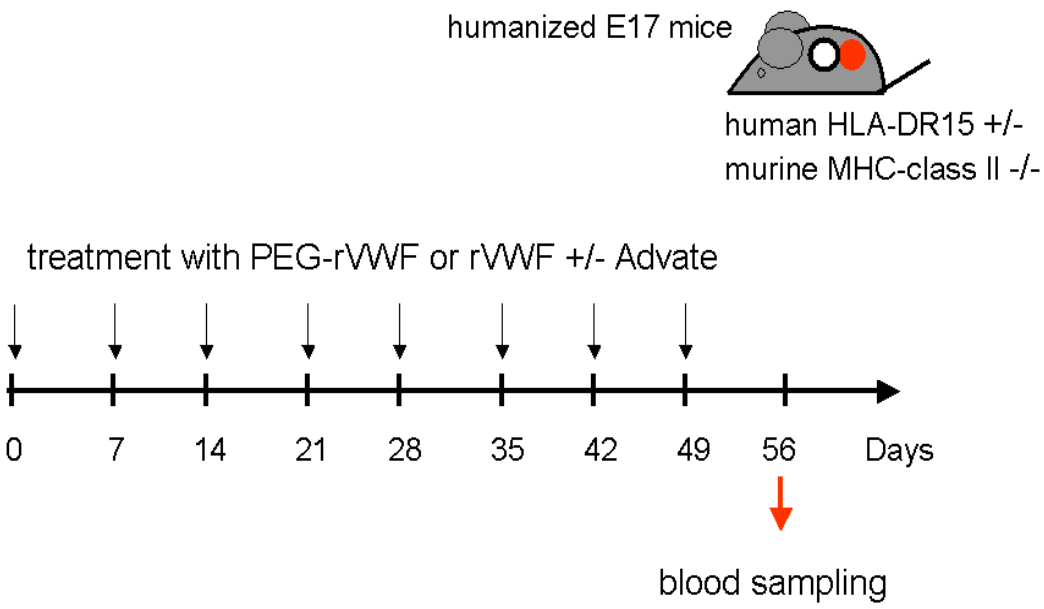

Figure 12: Treatment schedule for study 2: comparative immunogenicity of rVWF and PEG-rVWF in mice

Table 5: Study design for study 2: comparative immunogenicity of rVWF and PEG-rVWF in mice

Group	number	target dose	Group	number	target dose
1	1817 1822 1831 1832 1833 1839 1840 1841 1842 1848	7,5 µg rVWF (300 µg/kg) in 200µl i.v.	6	1792 1796 1797 1798 1799 1803 1804 1655 1660 1669	2,5 IU (100 IU/kg) Advate + 7,5 µg rVWF (300 µg/kg) in 200 µl
2	1849 1850 1852 1859 1867 1873 1874 1880 1883 1884	7,5 µg PEG-rVWF (300 µg/kg) in 200µl i.v.	7	1672 1673 1678 1679 1682 1686 1687 1688 1693 1700	2,5 IU (100 IU/kg) Advate + 7,5 µg PEG-rVWF (300 µg/kg) in 200 µl
3	1885 1891 1892 1893 1894 1895 1898 1903 1904 1909	15 µg rVWF (600 µg/kg) in 200µl i.v.	8	1702 1703 1739 1740 1741 1742 1749 1753 1755 1757	5,0 IU (200 IU/kg) Advate + 15,0 µg rVWF (600 µg/kg) in 200 µl
4	1912 1918 1919 1921 1928 1785 1935 1779 1783 1784	15 µg PEG-rVWF (600 µg/kg) in 200µl i.v.	9	1763 1766 1770 1591 1601 1612 1613 1616 1617 1619	5,0 IU (200 IU/kg) Advate + 15,0 µg PEG-rVWF (600 µg/kg) in 200 µl
5	1467 1471 1472 1473 1474 1480 1490 1498 1502 1503	200 µl Formulation Buffer	10	1638 1639 1640 1641 1643 1564 1572 1580 1581 1583	2,5 IU (100 IU/kg) Advate in 200 µl
			11	1538 1543 1544 1550 1551 1558 1559 1506 1512 1514	5,0 IU (100 IU/kg) Advate in 200 µl

3.2.8 Defining the cut off and validation of the anti-PEG detection assay in rat

The aim of these experiments were on the one hand to define the cut off in mice, that was needed to distinguish between positive and negative plasma samples. On the other hand the anti-PEG antibody detection assay in rat had to be validated to comply with quality requirements.

Cut off definition

To monitor potential antibody development it was necessary to define a cut off to be able to distinguish between a positive and a negative sample. For this purpose, blood was taken from 20 untreated rats and the plasma was screened using the anti-PEG antibody detection assay in different dilutions for anti-PEG antibodies. The experiment was repeated 5 times. The results of the 1:10 dilution were used to calculate the cut off with the software SPSS using the 95% percentile approach.

Validation

Aim of this study was to describe the assessment of a flow cytometry (FACS) based method to detect and to titer antibodies of the IgG subclass originating from rat plasma that are directed against polyethylene glycol (PEG). For this purpose, inter- and intra-assay variation, specificity and linearity was assessed to test the reliability and accuracy of the method.

For inter-assay variation positive and negative control items were analyzed on 10 different days. For intra-assay variation positive and negative control items were analyzed in 10 parallels on the same day. Test items were serially diluted till the last positively assessed dilution was found (titer endpoint). A monoclonal IgG anti-PEG antibody from rabbit was spiked with a dilution of 1:1024 into negative rat plasma and used as positive control. The antibody was conjugated before with a fluorochrome using the APC conjugation KIT from AbD Serotec. Pooled negative rat plasma was used as negative control.

Calculations

For each dilution, the respective median fluorescence intensity (MFI) provided the basis for evaluation. The dilution was assessed as positive if the MFI was ≥41,20 due to the definition of the cut off. The dilution was assessed as negative if the MFI was <41,20. The last dilution of the serial dilution analyzed that was found to be positive was defined as the titer endpoint. The titer endpoint that was most common in all analysis was defined as the mode titer.

For assessment of specificity of the assay, positive and negative control items were analyzed following pre-incubation with soluble PEG or with PBS. Samples were measured at an initial 1:20 dilution but not serially diluted to identify the corresponding titer endpoint. Samples were compared at a 1:20 dilution based on their MFI signal. Since the MFI signals were the raw data to calculate the corresponding titer endpoint, this procedure reflected also a reliable approach to prove specificity. The acceptance criteria was re-defined as follows:

- Positive control items should be assessed as positive.
- Negative control items need to be assessed as negative.
- Pre-incubation of positive control items should result in a negative assessment of the sample.

Pre-incubation of negative control items should have no impact on assessment.

3.2.9 Monitoring of potential anti-PEG antibody developing in rats treated with PEGylated proteins

The purpose of this study was to evaluate the potential toxicity of a PEGylated recombinant factor VIII (rFVIII) administered intravenously in rats every other day for 30 days (15 doses). This study was conducted in accordance with FDA Regulations for Good Laboratory Practice (GLP), 21 CFR Part 58 (Ref 1).

Table 6: Study design for 30-day intravenous toxicity study in rats

Group	Article	Target Dose (mg/kg/dose)	Volume (mL/kg/dose)	Animal Numbers	
				Males	Females
1	ADVATE (rFVIII)	0,4	20	129-140	177-188
2	Formulation buffer	0	5	141-152	189-200
3	PEG-rFVIII	0,4	5	153-164	201-212
4	PEG-rFVIII	0,8	5	165-176	213-224

Dose justification and administration

Based on a maximum weekly clinical dose of ADVATE of 160 IU/kg administered 5 times/week (800 IU/kg/week) and specific activity ranging from 4000 – 10000 IU/mg protein, the maximum weekly clinical dose of rFVIII an a protein basis is 0,2-0,08 mg/week. The targeted high-dose for PEG-rFVIII of 0,8 mg/kg/dose every other day or approximately 3,5 times a week represents a weekly rFVIII dose of 2,8 mg/kg/week on a protein basis. Therefore, the targeted high dose of PEG-rFVIII is 14-35 times higher than the proposed maximum clinical dosage.

The targeted dose for ADVATE, the reference control article, of 0,4 mg/kg/dose or 7 times/week represents a weekly rFVIII dose of 2,8 mg/kg/week on a protein basis, which is equivalent to the high dose test article.

The calculated volume of test or control article was administered intravenously via a tail vein at a rate of approximately 2 mL/min. The intravenous route of administration was selected for this study because it is the intended route of human exposure in the clinical situation. Doses were adjusted weekly based on changes in body weight.

Blood was collected prior to dosing and after the last dose. Blood samples were processed for collection of plasma and analyzed for anti-PEG antibodies.

Each sample was analyzed two times using the anti-PEG detection assay to define the FACS titer endpoint. The titer endpoint is given in log 2 titer

values and the average from both runs was calculated. Then the FACS titer log 2 values from pre- and post - immunization were compared. A sample was evaluated positive when the difference between log 2 titer values from pre-immunization and from post-immunization is ≥ 2.

A monoclonal IgG anti-PEG antibody from rabbit was spiked with a dilution of 1:1024 into negative rat plasma was used as positive control. The antibody was conjugated before with a fluorochrome using the APC conjugation KIT from AbD Serotec. Pooled negative rat plasma was used as negative control.

3.2.10 Experimental set up for screening human plasma samples for natural anti-PEG antibodies

The aim of this study was to evaluate the incidence of anti-PEG antibodies within a healthy human population. This study in healthy blood donors provides important information for the screening of patients to be included in clinical studies of the new drug candidates.

The human plasma samples from healthy donors are provided by Baxter Plasma Sourcing. The samples were 1:10 pre-diluted with PBS to obtain a final concentration of 1:20 within the anti-PEG antibody detection assay. It is not possible to define a clear cut off due to the fact that there are no human individuals that have never been exposed to PEG. Therefore a different way of distinction between positive and negative samples had to be found. Each plasma sample was analyzed twice: First the samples were pre-incubated with 5µl of soluble PEG (wt 200) for 30 minutes at room temperature, then the other samples were incubated with 5µl PBS. Plasma samples that showed a significant reduction in their MFI value when pre-incubated with soluble PEG in comparison to pre-incubation with PBS, were counted as positive.

A monoclonal rabbit IgG anti-PEG antibody was used as a positive control. Unstained beads and the beads incubated with the secondary antibody only and soluble PEG were taken as negative control.

4. Results

4.1 Establishing the anti-PEG antibody detection assay

4.1.1 First experiments

A protein A purified IgG antibody from rabbits that were immunized before with PEG-FVIII was taken as a positive control while establishing the anti-PEG antibody detection assay. Figure 13 shows the positive control after a serially dilution with PBS was done. Starting with a 1:2 dilution (rabbit charge 1 IgG 1), 8 dilutions were done.

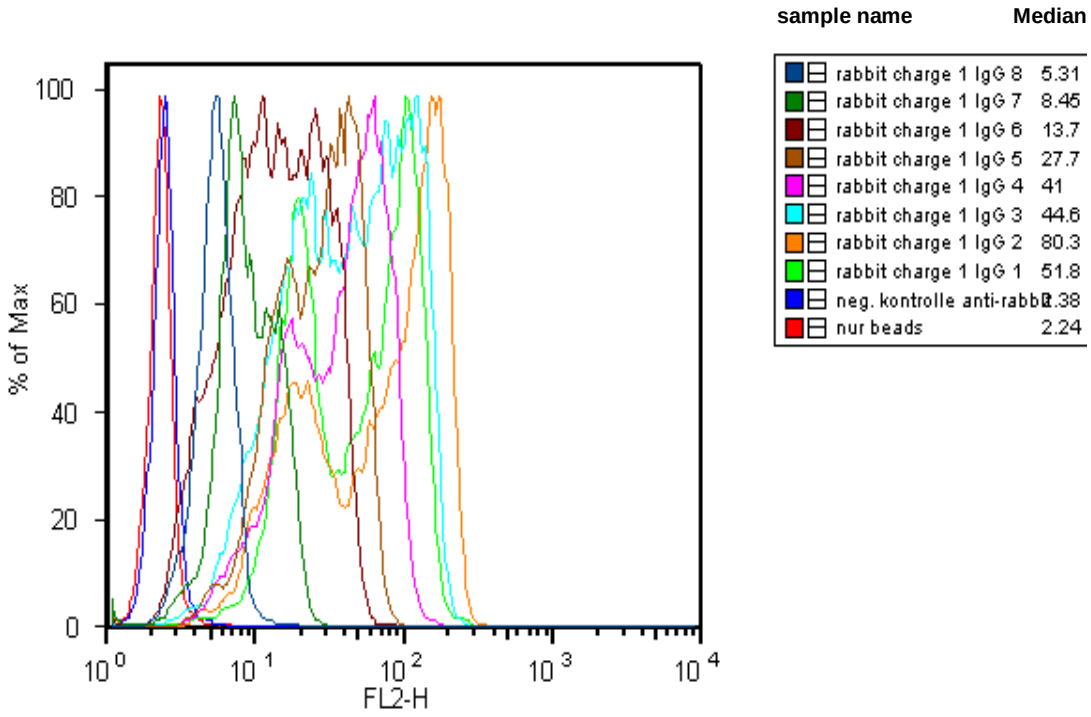

Figure 13: anti-PEG antibody detection with a protein A purified rabbit IgG antibody

Concentration of the beads

To see whether the concentration of the beads has an influence on the detection of the anti-PEG antibodies or not, a titration of the beads was done.

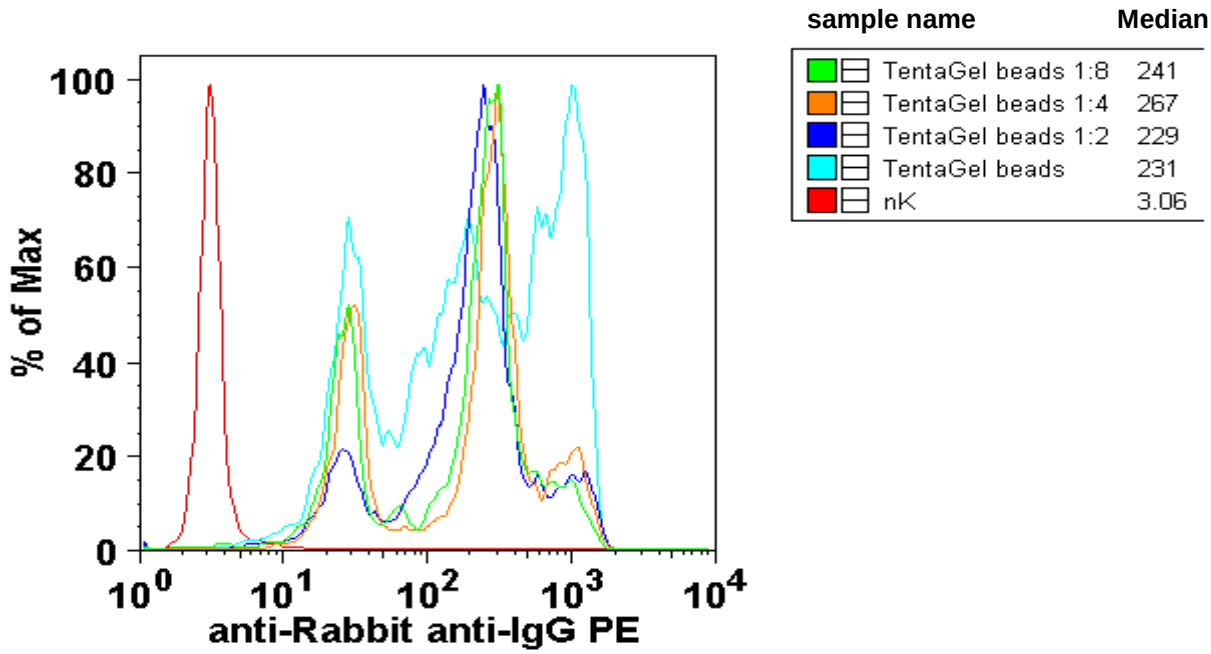

Figure 14: Influence of the concentration of TentaGel beads on the detection of anti-PEG antibodies

Titration of monoclonal anti-PEG antibodies (IgG, IgM) and of secondary antibodies in mouse

The aim of this experiment was to find out the best dilution for the antibodies used for analyses. Lowering the concentration of the antibody to a point that would be necessary to see a positive signal, enabled us to exclude unspecific binding on the one hand, and on the other hand reduce the costs of each experiment. Figure 15 and Figure 16 show examples of the titration for the monoclonal IgM anti-PEG antibody. The combination of an 1:32 dilution of the primary antibody together with a dilution of 1:32 of the secondary antibody showed in all experiments the most stable positive signal with the lowest concentration.

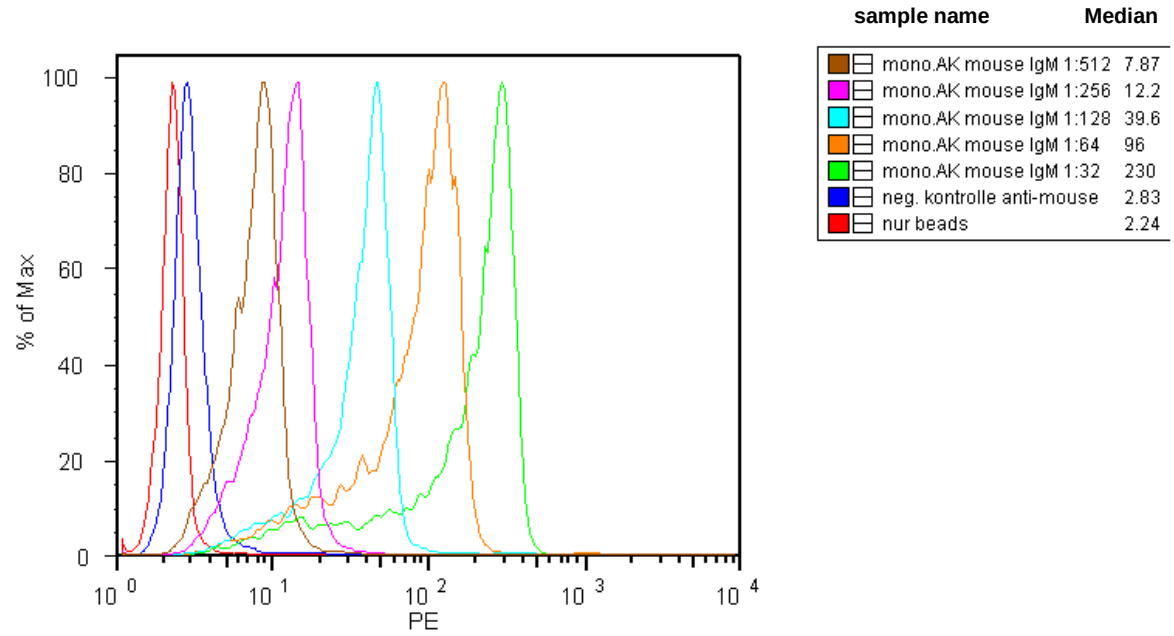

Figure 15 : Titration of a monoclonal IgM antibody directed against polyethylene glycol

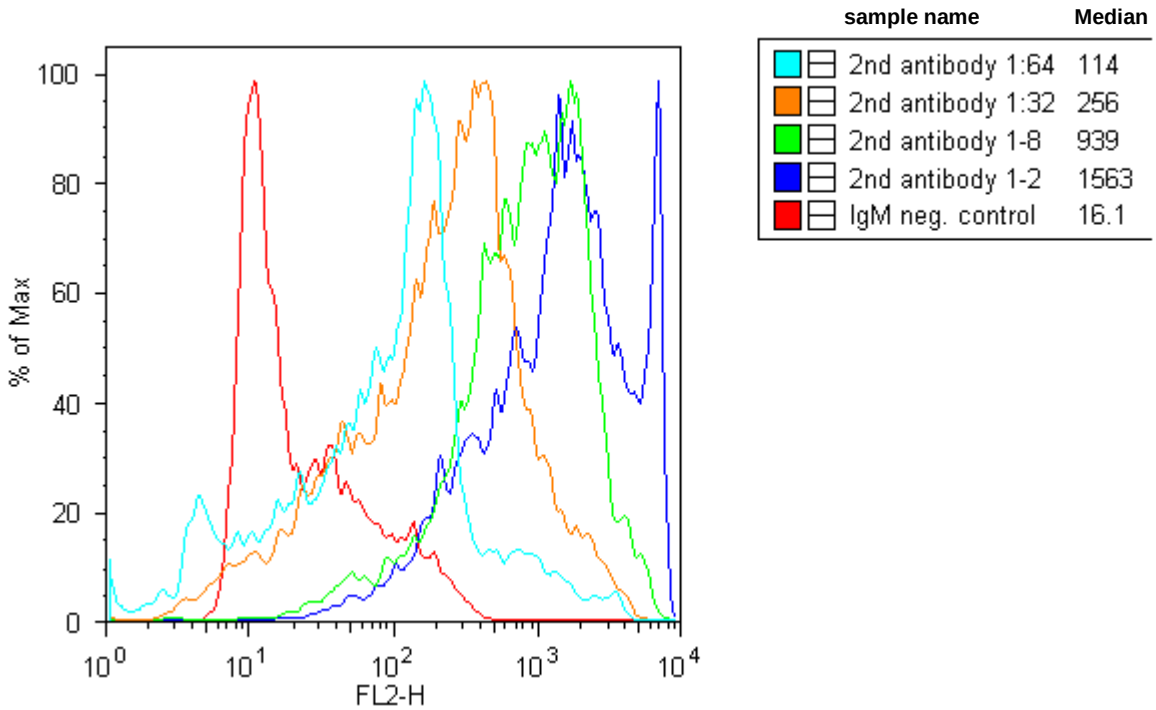

Figure 16: Titration of the secondary antibody with a dilution of the primary antibody of 1:32

4.1.2 Comparison of TentaGel beads and TentaGel beads coupled to branched PEG

TentaGel resins are grafted copolymers consisting of a low cross-linked polystyrene matrix on which polyethylene glycol is grafted. The copolymer contains about 50 - 70 % PEG (w/w). Therefore, the properties of these polymers are highly dominated by the properties of PEG and no longer by the polystyrene matrix (Rapp Polymer GmbH). However, the polyethylene glycol that was used in this approach was linear, but the polyethylene glycol used for PEGylation of recombinant factor FVIII had a branched chain. Therefore the TentaGel beads were additionally PEGylated with our branched PEG reagent using the same PEGylation reaction as used for the PEGylation of recombinant factor VIII. The additional PEGylation should cover all possible epitopes, and therefore all possible antibodies directed against PEG, within the test. To study if the additional PEGylation had an influence on the anti-PEG antibody detection assay, a comparison with untreated TentaGel beads using plasma sample of mice that contained anti-PEG antibodies was done.

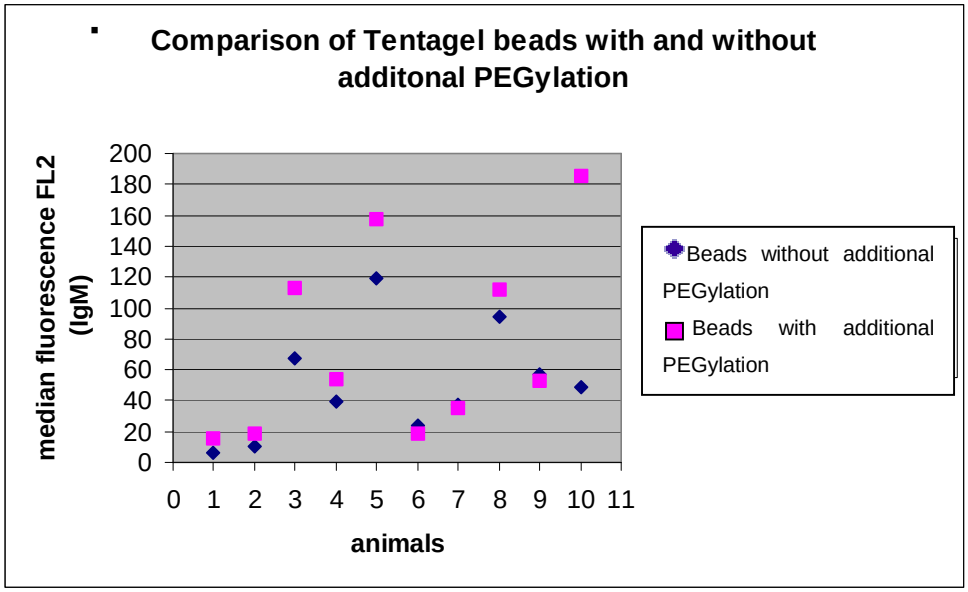

Figure 17: Comparison of TentaGel beads with and without additional PEGylation, using mice plasma samples. The mice have been treated intravenously with PEGylated protein.

As shown in figure 17 the additional PEGylation had an influence on the assay. The samples tested for anti-PEG antibodies using beads coupled to branched PEG showed the same or even a higher median fluorescence intensity. Moreover beads coupled to branched PEG showed a reduced auto fluorescence (figure 18) and were easier to handle because of a reduced tendency to aggregate.

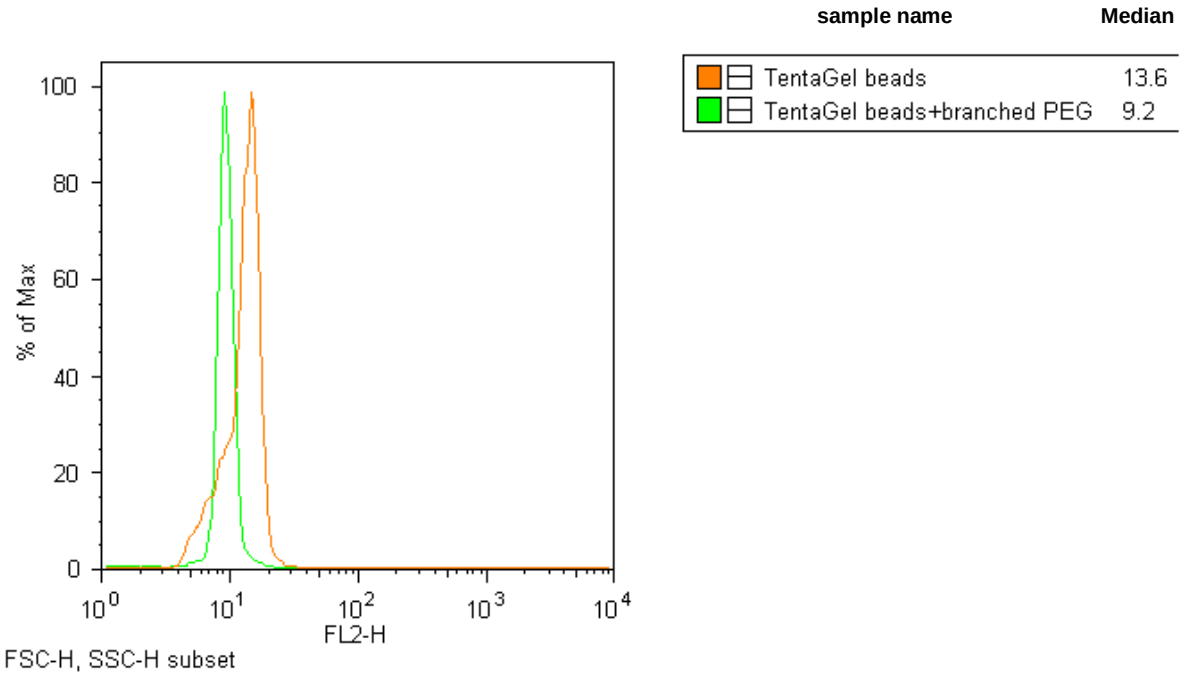

Figure 18: Analyses of auto fluorescence comparing TentaGel beads with and without additional PEGylation.

4.1.3 Storage and stability of the beads

Another important issue was to ensure that the beads used for analyses are stable, first of all in terms of PEGylation, and how to store them best without destabilizing. To encounter that question, one lot of the beads was stored under 4℃ and tested several times during a time period of 4 weeks. In addition three different batches of beads with additional PEGylation were produced and lyophilized. Every batch was stored under 3 different conditions, namely 4℃, room temperatur e and 37℃. Analyses were done shortly after production and after 4 weeks of storing (figure 20).

As you can see in figure 19 the beads showed no significant change in their median fluorescence intensity. However, to ensure that the beads of the same lot would be stable during a whole preclinical or clinical study, they got lyophilized after PEGylation reaction with the branched reagent.

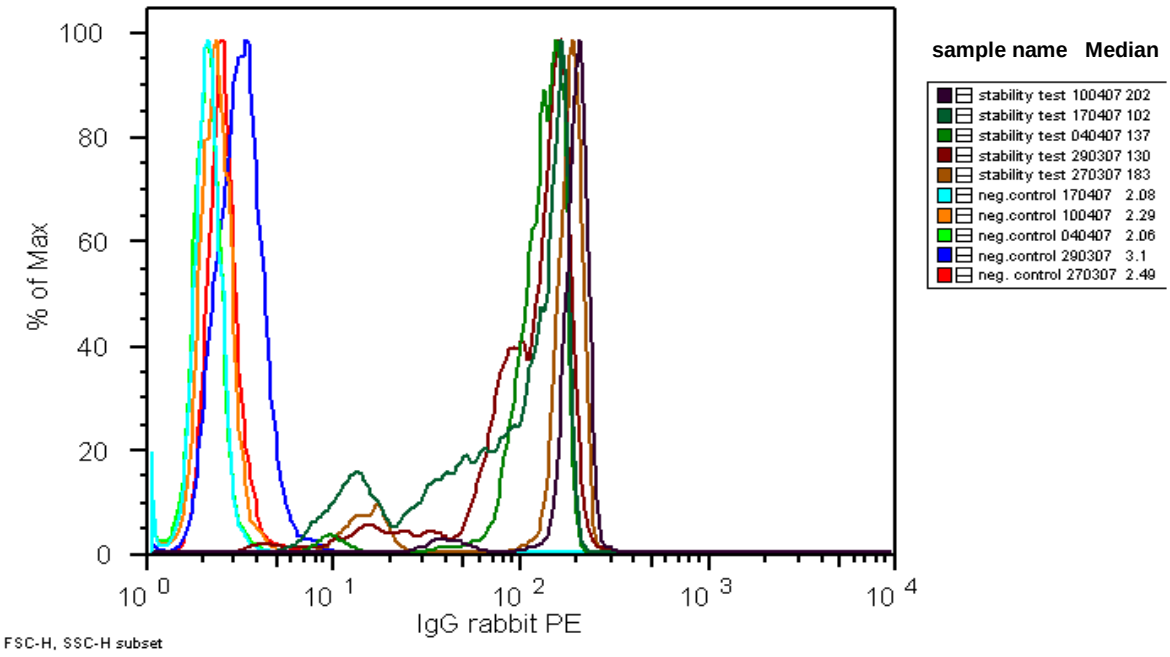

Figure 19: Storage of TentaGel beads under 4℃ and analyzed s everal times within one month with a protein A purified anti-PEG antibody from rabbit.

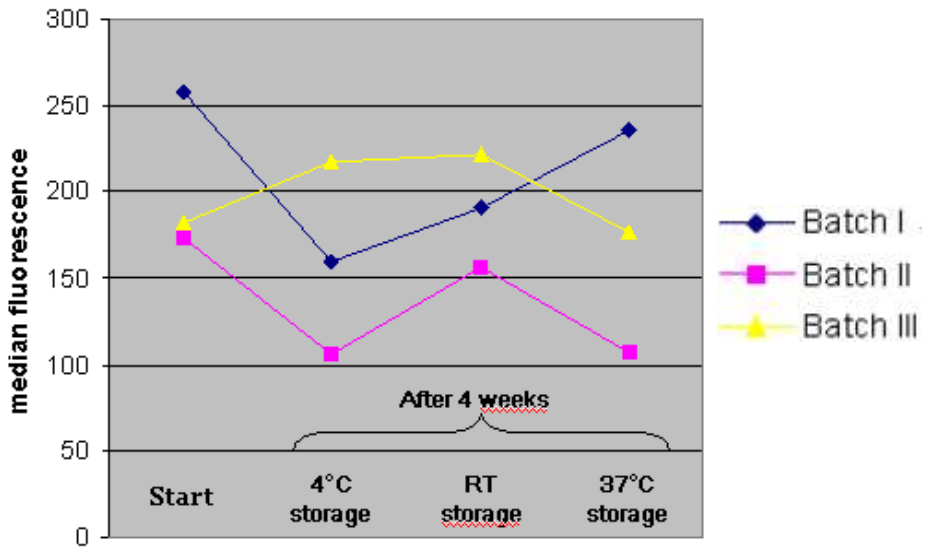

Figure 20: Comparison of different batches of TentaGel beads with additional PEGylation; all batches were stored for 4 weeks under 3 different conditions, 4℃, room temperature and 37℃. The beads were tested with a protein A pu rified anti-PEG antibody from rabbit, before and after storage.

4.1.4 Competition Assay

To study whether the antibodies contained in our plasma samples are specific for PEG, a competition assay was done. As you can clearly see in figure 21, the positive control alone had a very high median fluorescence intensity (MFI) of 2938. When you pre-incubate with the soluble PEG the MFI goes down to 113, and up again if you dilute the soluble PEG before pre-incubation.

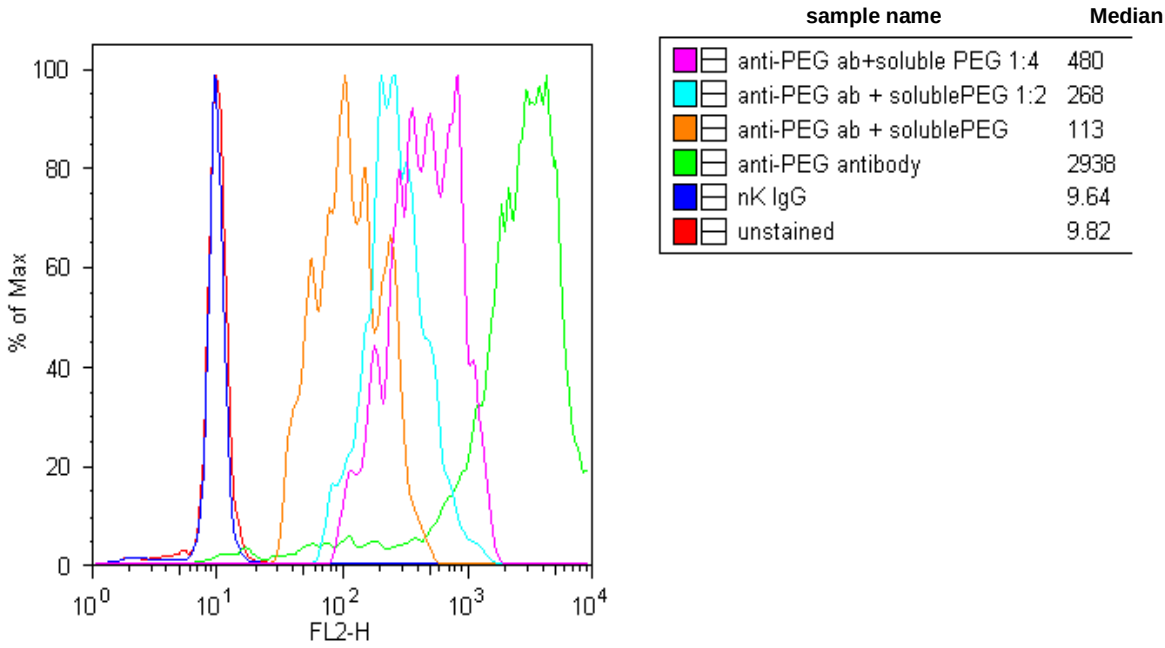

Figure 21: Competition Assay: A monoclonal anti-PEG antibody was pre-incubated with a soluble PEG (wt=200) in different dilutions, before they were mixed together with the TentaGel beads.

4.2 Anti-PEG antibodies in mouse

4.2.1 Defining the cut off

To monitor potential antibody development it was necessary to define a cut off to be able to distinguish between a positive and a negative sample. For this purpose, blood was taken from 53 untreated mice and the plasma was screened in different dilutions for anti-PEG antibodies.

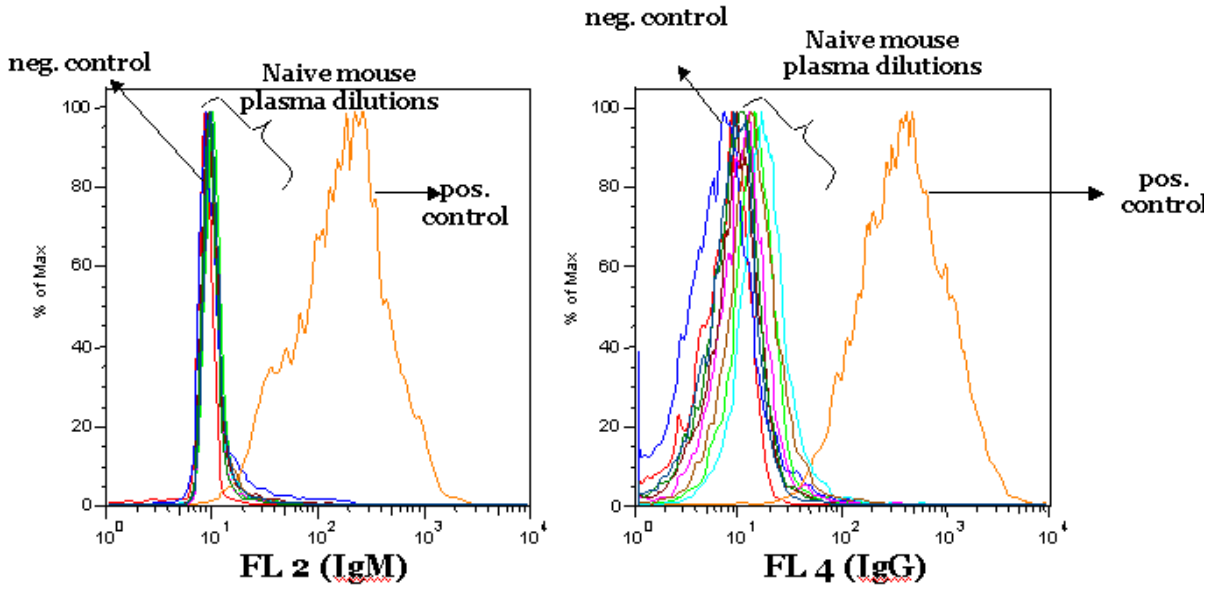

Figure 22: Titration analyses of plasma from a naïve mouse. The positive control was a monoclonal anti-mouse anti-PEG antibody.

For the cut off analyses only the values from the plasma with a 1:20 dilution were taken.

Table 7: Analyses of plasma from untreated mice in a 1:20 dilution.

Cut off definition for anti-PEG antibody in mouse

plasmadilution: 1/20

	MFI PE (IgM)	MFI APC (IgG)		MFI PE (IgM)	MFI APC (IgG)
animal 1	8,33	1,37	animal 31	9,25	2,19
animal 2	8,2	1,37	animal 32	9,82	1,76
animal 3	8,17	1,4	animal 33	10,1	2,05
animal 4	8,08	1,36	animal 34	9,29	2,59
animal 5	7,87	1,4	animal 35	9,95	1,93
animal 6	10,3	2,09	animal 36	6,07	1,83
animal 7	10,8	1,73	animal 37	7,7	2,08
animal 8	10,9	1,69	animal 38	6,7	1,29
animal 9	9,85	1,64	animal 39	7,36	1,14
animal 10	9,88	1,62	animal 40	6,72	1,08
animal 11	11,7	1,68	animal 41	6,88	1,04
animal 12	10,5	1,73	animal 42	6,92	1,04
animal 13	8,26	1,78	animal 43	6,87	1,03
animal 14	8,33	1,69	animal 44	6,99	1,01
animal 15	7,98	1,66	animal 45	7,57	1,04
animal 16	8,37	1,75	animal 46	8,98	1,14
animal 17	7,74	1,83	animal 47	9,13	1,05
animal 18	7,87	1,88	animal 48	8,9	2,12
animal 19	8,85	1,72	animal 49	9,04	2,7
animal 20	9,15	2,83	animal 50	8,62	2
animal 21	8,78	3,28	animal 51	8,66	2,34
animal 22	8,88	2,72	animal 52	9,01	2,39
animal 23	9,63	2,49	animal 53	8,77	2,37
animal 24	9,35	2,55			
animal 25	9,85	2,78			
animal 26	9,35	2,67			
animal 27	9,83	2,59			
animal 28	9,1	1,93			
animal 29	9,4	1,85			
animal 30	10,2	1,89			

MFI = Median Fluorescence Intensity

From the results presented in table 7 the 95% percentile was calculated and defined as cut off.

Percentile

		Percentile						
		5	10	25	50	75	90	95
Weighted average	Median FL2 (IgM)	6,7140	6,8960	7,9250	8,8800	9,7250	10,2600	10,8300

Figure 23A

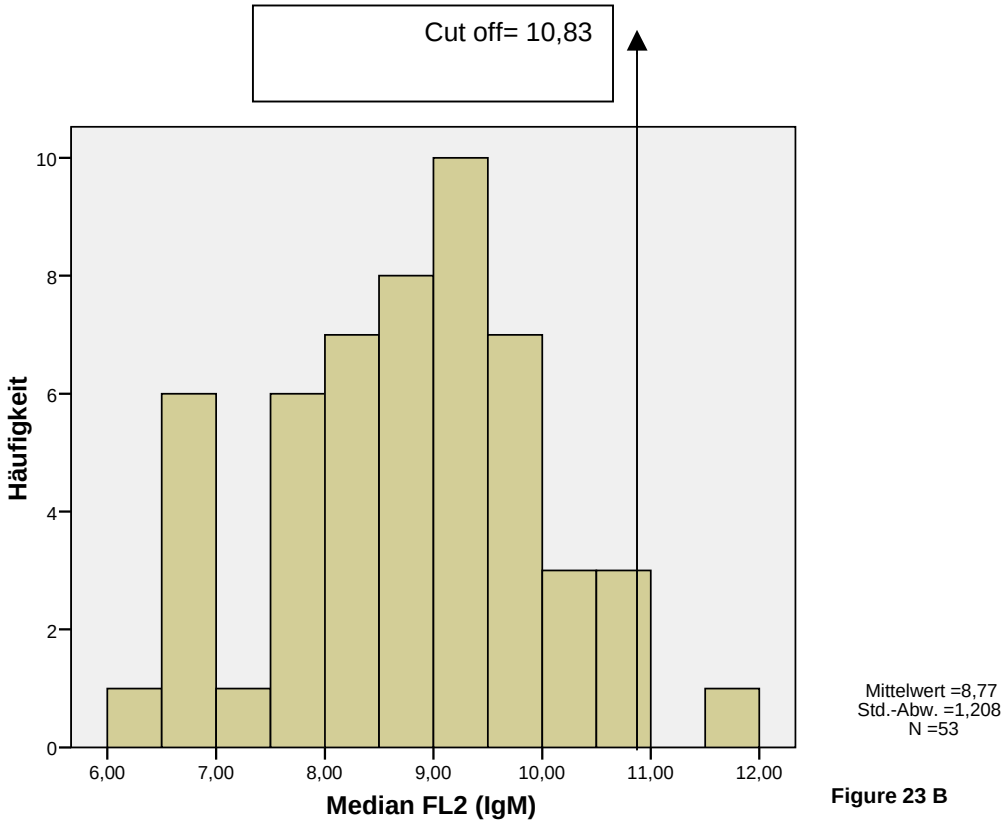

Figure 23 B

Figure 23: Calculation of the 95% percentile by SPSS (Figure 23A) for mouse IgM anti-PEG antibody MFI values and a histogram showing the absolute frequency of all values in the data set(Figure 23B).

Percentile

		Percentile						
		5	10	25	50	75	90	95
Weighted average	MFI APC (IgG)	1,0370	1,0440	1,3850	1,7800	2,2650	2,6880	2,7950

Figure 24A

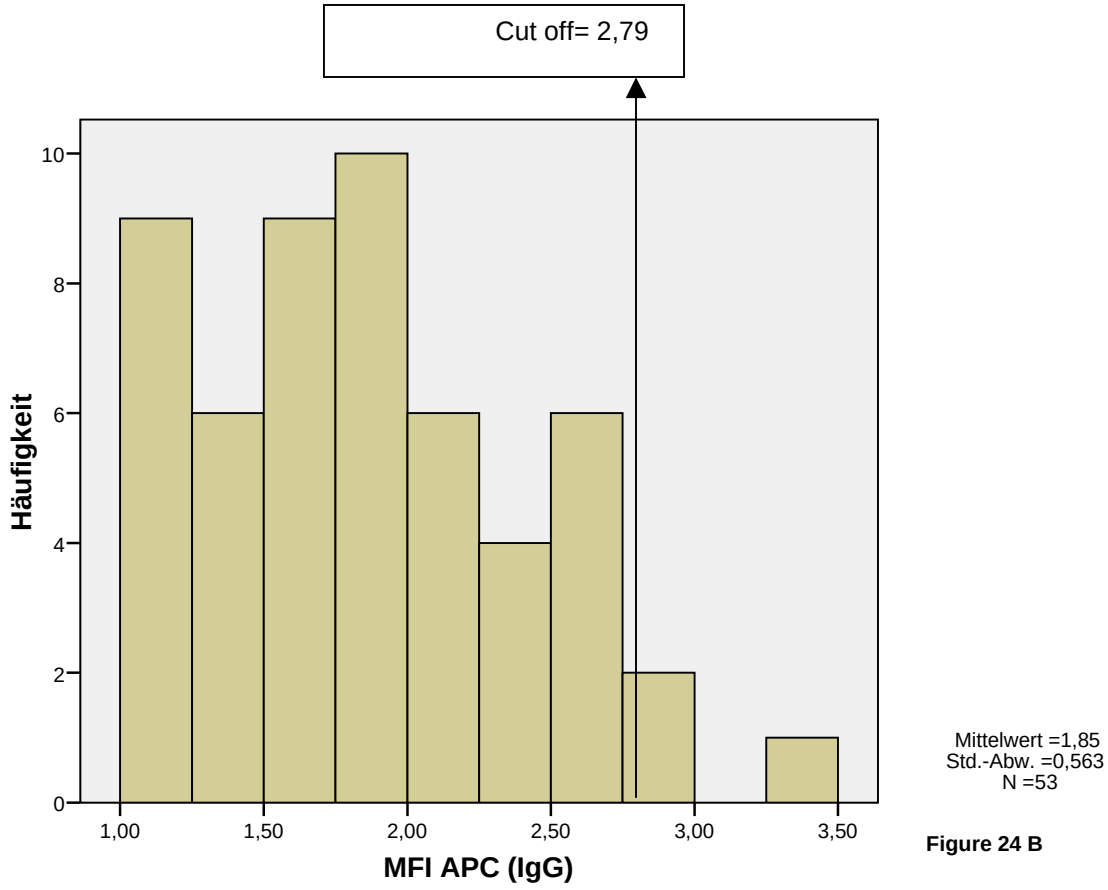

Figure 24 B

Figure 24: Calculation of the 95% percentile by SPSS (Figure 24A) for mouse IgG anti-PEG antibody MFI values and a histogram showing the absolute frequency of all values in the data set (Figure 24B)

4.2.2 Monitoring of potential anti-PEG antibody developing in mice treated with PEGylated proteins

The anti-PEG antibody detection assay was used to monitor the development of anti-PEG antibodies in several preclinical studies of PEGylated protein product candidates. In the course of the factor VIII Half-life (FVIII-HL) project (extension of the factor VIII half-life), von Willebrand

factor (VWF) was PEGylated. VWF is the carrier molecule of FVIII. The aim of series 1 and series 2 in mice was to compare the immunogenicity of recombinant von Willebrand factor (rVWF) and PEGylated rVWF in humanized E17 mice.

Comparative immunogenicity of rVWF and PEG-rVWF in humanized E17 mice: study 1

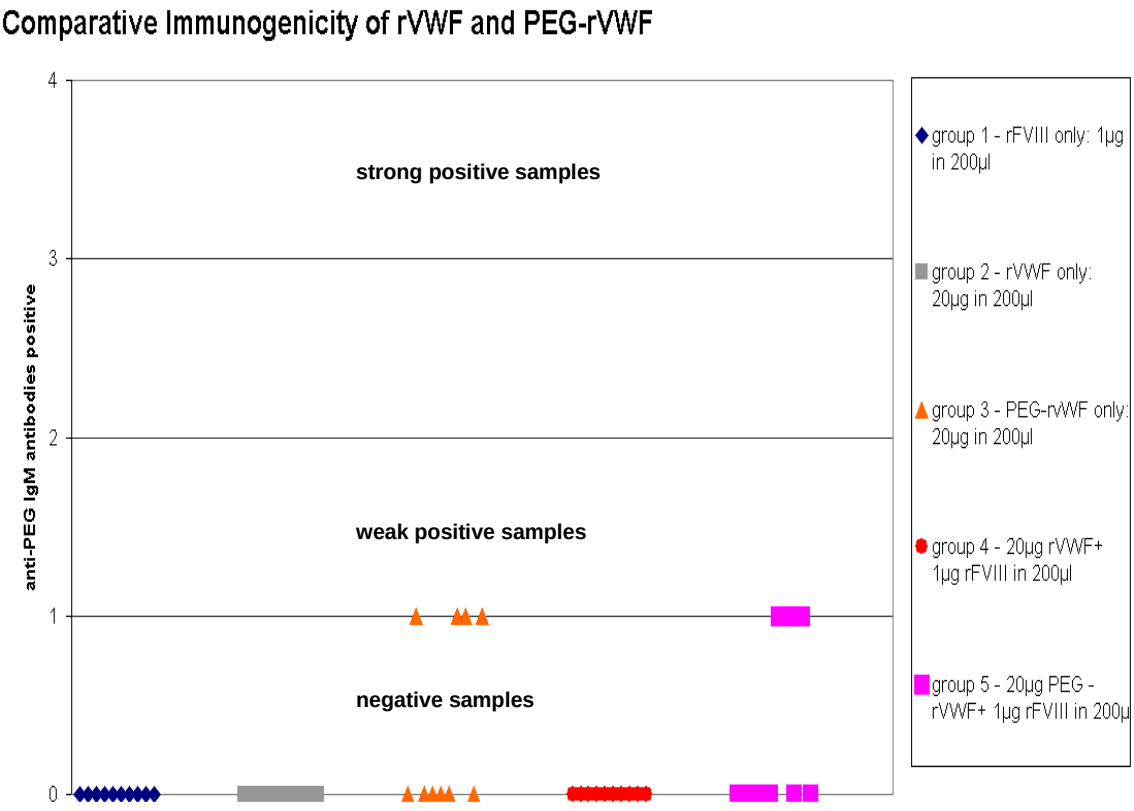

Figure 25: Development of IgM anti-PEG antibodies during the comparative immunogenicity study of rVWF and PEG-rVWF: study 1

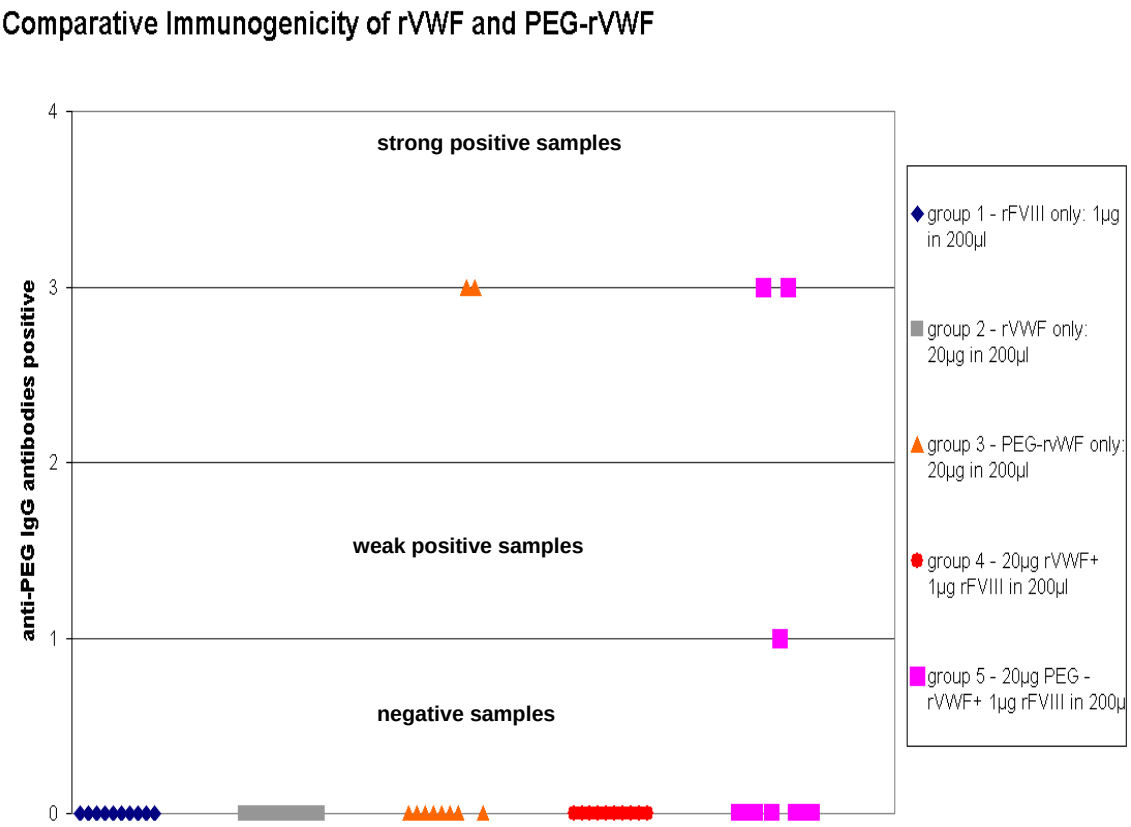

Figure 26: Development of IgG anti-PEG antibodies during the comparative immunogenicity study of rVWF and PEG-rVWF: study 1

Comparative immunogenicity of rVWF and PEG-rVWF in humanized E17 mice: study 2

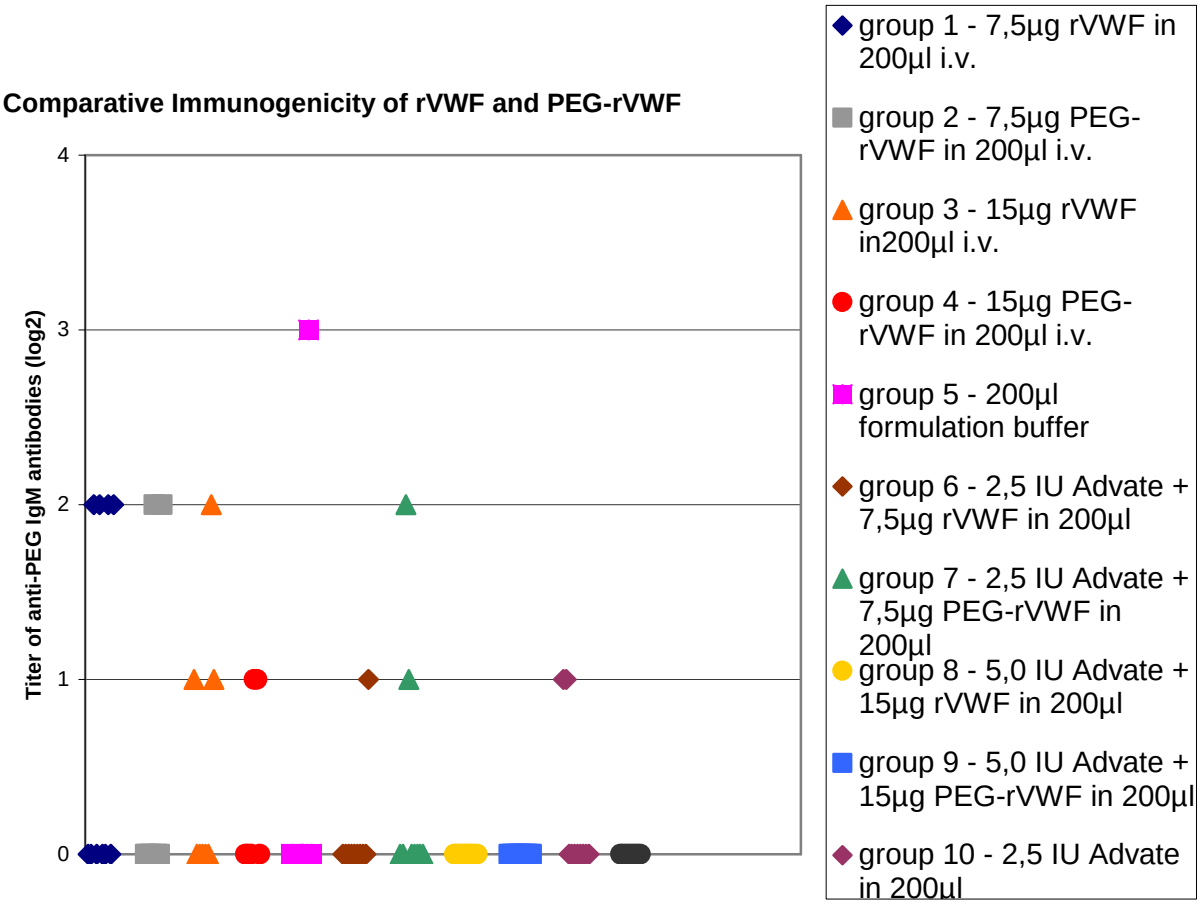

Figure 27: Development of anti-PEG IgM antibodies during the comparative immunogenicity study of rVWF an PEG-rVWF in humanized E17 mice: study 2. The diagram shows the anti-PEG IgM antibodies as log 2 FACS Titer values.

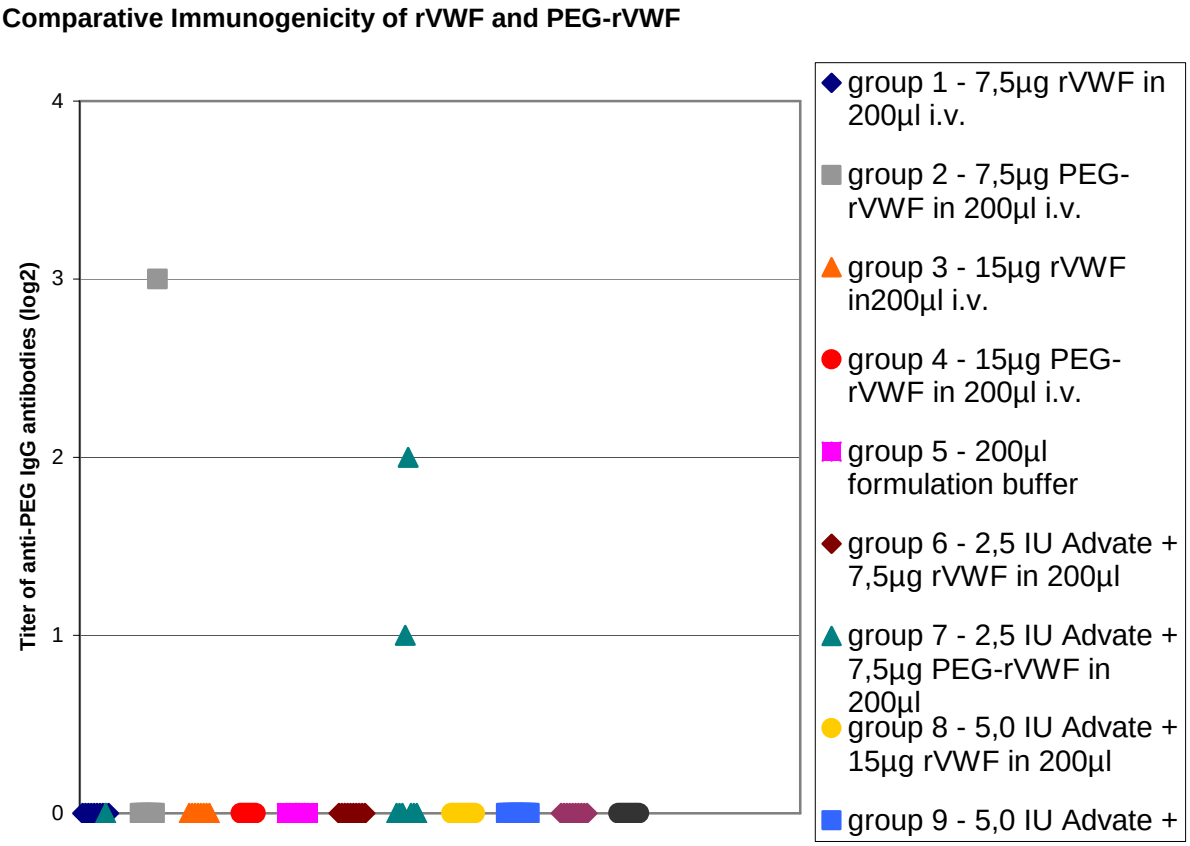

Figure 28: Development of anti-PEG IgG antibodies during the comparative immunogenicity study of rVWF an PEG-rVWF in humanized E17 mice: study 2. The diagram shows the anti-PEG IgM antibodies as log 2 FACS Titer values.

4.3 Anti-PEG antibodies in rat

4.3.1 Defining the cut-off

To monitor potential anti-PEG antibody development in rats, it was necessary to define a cut off to be able to distinguish between a positive and a negative sample. For this purpose, blood was taken from 20 untreated rats and the plasma was screened in different dilutions for anti-PEG antibodies. The same samples were analyzed on 5 different days.

Table 8: Analyses of plasma from untreated rats in a 1:10 dilution with 5 repeats.

Cut-Off definition for anti-PEG antibody detection assay in rat
citrated rat plasma obtained from untreated rats

Dilution 1/10

Male	Replica 1 MFI APC (IgG)	Replica 2 MFI APC (IgG)	Replica 3 MFI APC (IgG)	Replica 4 MFI APC (IgG)	Replica 5 MFI APC (IgG)
1	42,8	28,8	40	35,4	34,6
2	39,5	27,7	42,6	32,7	35,6
3	34,1	27	39,7	32,9	39,2
4	41,2	27,4	40,8	32,6	34,6
8	37,1	26,2	40,6	33,4	40,4
9	39,4	25,8	36,4	32,5	35,5
10	41,1	28,1	37,8	32,5	35,2
18	37,7	23,3	38,4	31,2	36,9
19	35,5	21,2	37,9	32,8	35,4
20	39,8	27,5	42	32,9	34,7
Female					
5	35,8	25,1	38,1	31,0	34,3
6	34,1	24,3	37,9	30,5	34,0
7	40	25,3	43,3	32,6	33,8
11	37,3	25,9	36,4	31,0	34,2
12	37,8	27,3	35,1	31,3	33,9
13	34	25,8	37,2	30,7	34,6
14	34,5	25,7	36,8	31,1	35,0
15	34,2	33,7	38	32,7	36,5
16	38,3	33,4	34,1	32,5	34,3
17	36,1	25,9	33,4	32,4	34,6
unstained	33,4	25,4	39,6	33,8	34,1

The results of this assay were used to calculate the cut off with the software SPSS using the 95% percentile approach.

Percentile

		Percentile						
		5	10	25	50	75	90	95
weighted average	Replica 1	25,32	25,93	31,58	34,30	37,60	40,00	41,20

Figure 29A

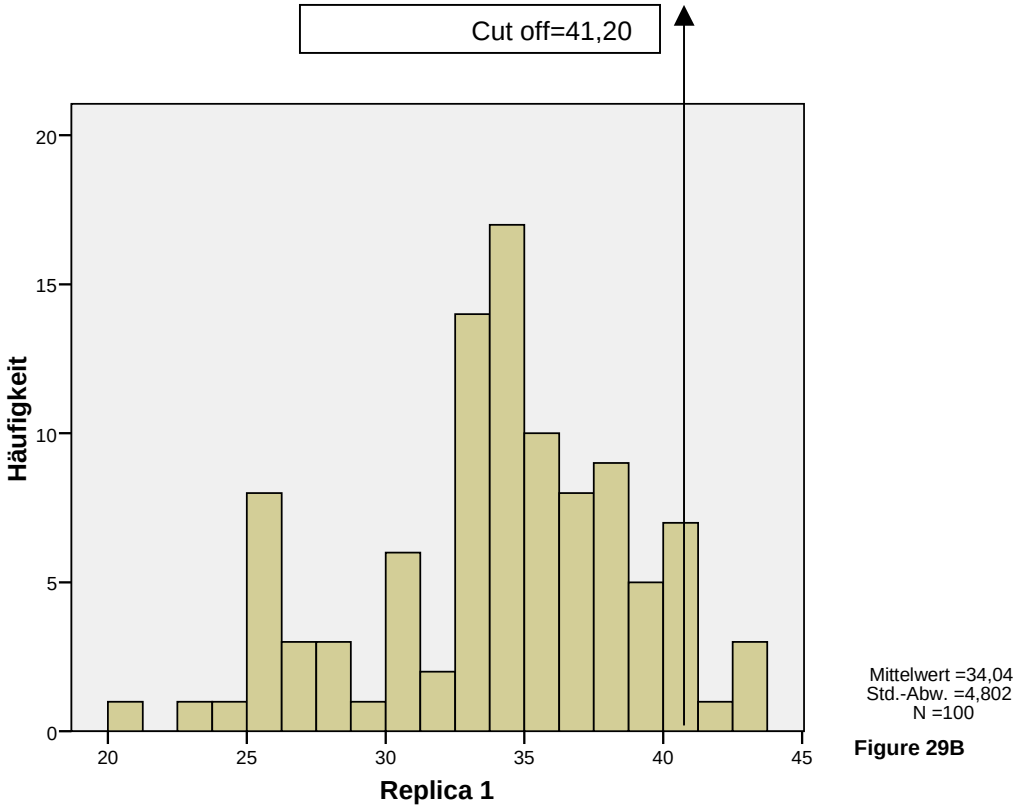

Figure 29B

Figure 29: Calculation of the 95% percentile by SPSS (Figure 29A) for rat IgG anti-PEG antibody MFI values and a histogram showing the absolute frequency of all values in the data set (Figure 29B).

4.3.2 Validation of the anti-PEG antibody detection in rat for further studies under GLP-conditions

The aim of this study was to describe the assessment of a flow cytometry (FACS) based method to detect and to titer anti-PEG antibodies of the IgG subclass originating from rat plasma. For this purpose inter-assay variation (table 9), intra-assay variation (table 10), specificity (table 11) and linearity (figure 29) for this method were obtained.

Inter-assay variation

Table 9 : Inter-assay variability (titer endpoints given as log 2 titer values)

Interassay		
run	negative control item	positive control item
1	nd	4
2	nd	4
3	nd	3
4	nd	3
5	nd	3
6	nd	3
7	nd	4
8	nd	4
9	nd	3
10	nd	3
nd=not detectable: MFI< 41,20 at 1:20 dilution		

Intra-assay variation

Table 10: Intra-assay variability (titer endpoint given as log 2 titer values)

Intraassay		
run	negative control items	positive control items
1	nd	4
2	nd	3
3	nd	3
4	nd	4
5	nd	4
6	nd	3
7	nd	3
8	nd	4
9	nd	4
10	nd	3
nd=not detectable: MFI< 41,20 at 1:20 dilution		

Specificity

Table 11: Specificity (MFI values given)

Specificity			
negative control item		positive control item	
without antigen	with antigen	without antigen	with antigen
nd	nd	151	nd
not detectable: MFI< 41,20 at 1:20 dilution			

Linearity

The relationship between antibody titer and MFI values was characterized graphically.

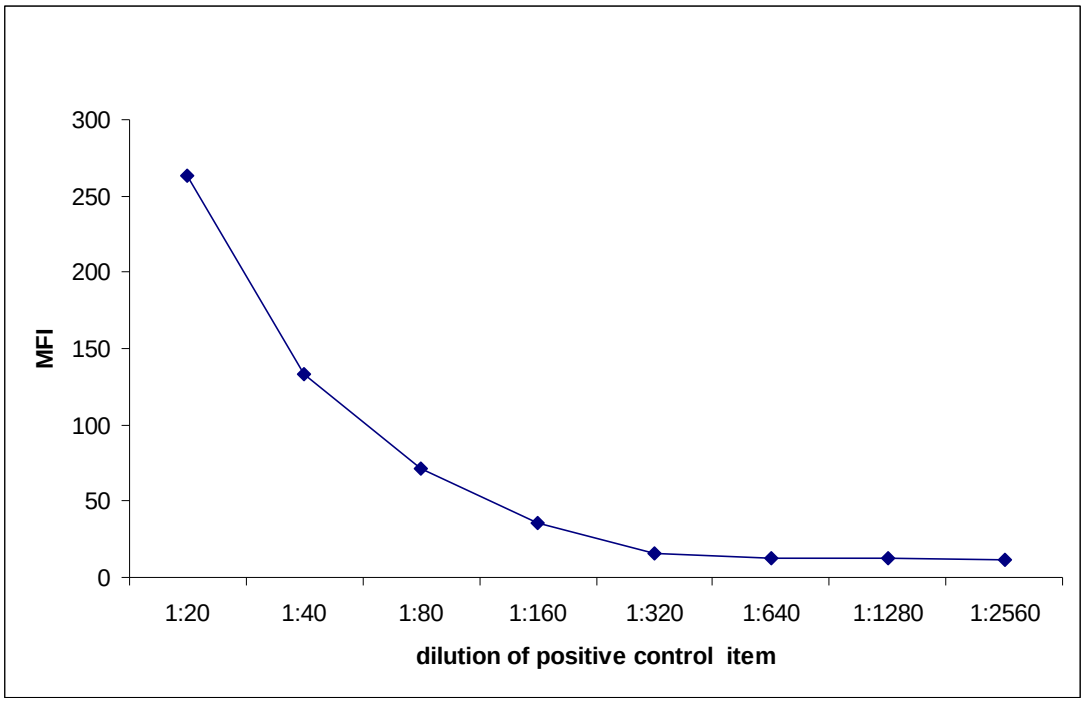

Figure 30: Linearity between antibody titers and MFI values.

4.3.3 Monitoring of potential anti-PEG antibody developing in rat

The purpose of this study (table 12: study design) was to evaluate the potential toxicity of a PEGylated recombinant factor VIII (rFVIII) administered intravenously in rats every other day for 30 days (15 doses). This study was conducted in accordance with FDA Regulations for Good Laboratory Practice (GLP), 21 CFR Part 58 (Ref 1).

Table 12: Study design

Group	Article	Target Dose (mg/kg/dose)	Volume (mL/kg/dose)	Animal Numbers	
				Males	Females
1	ADVATE (rFVIII)	0,4	20	129-140	177-188
2	Formulation buffer	0	5	141-152	189-200
3	PEG-rFVIII	0,4	5	153-164	201-212
4	PEG-rFVIII	0,8	5	165-176	213-224

Blood was collected prior to dosing and after the last dose. Blood samples were processed for collection of plasma and analyzed for anti-PEG antibodies.

Each sample was analyzed two times using the anti-PEG detection assay to define the FACS titer endpoint. The titer endpoint is given in log 2 titer values and the average from both runs was calculated. Then the FACS titer log 2 values from pre- and post - immunization were compared. A sample was evaluated positive when the difference between log 2 titer values from pre-immunization and from post-immunization is ≥ 2.

Table 13: Results from the 30-days intravenous study in rats. For each blood sample, 2 analyses have been done. The titer endpoint is given in log 2 titer values and the average from both runs was calculated. A sample is positive when the difference between log 2 titer values from pre-immunization and from post-immunization is ≥2.

animal	sex	Pre titer (1)	log 2	Pre titer (2)	log 2	average	Post titer (1)	log 2	Post titer (2)	log 2	average	pre-post	results
130	m	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
131	m	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
133	m	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
134	m	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
135	m	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
177	f	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
178	f	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
179	f	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
180	f	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
181	f	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
147	m	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
148	m	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
149	m	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
150	m	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
151	m	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
189	f	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
190	f	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
191	f	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
192	f	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
193	f	<20	0	<20	0	0	<20	0	<20	0	0	0	negative
156	m	<20	0	<20	0	0	>20	0	>20	0	0	0	negative
157	m	<20	0	<20	0	0	>20	0	>20	0	0	0	negative
158	m	<20	0	<20	0	0	>20	0	>20	0	0	0	negative
159	m	<20	0	<20	0	0	>20	0	>20	0	0	0	negative
160	m	<20	0	<20	0	0	>20	0	>20	0	0	0	negative
201	f	<20	0	<20	0	0	>20	0	>20	0	0	0	negative
202	f	<20	0	<20	0	0	>20	0	>20	0	0	0	negative
203	f	<20	0	<20	0	0	>20	0	>20	0	0	0	negative
204	f	<20	0	<20	0	0	40	2	>20	0	1	1	negative
205	f	<20	0	<20	0	0	40	2	40	2	2	2	positive
171	m	<20	0	<20	0	0	>20	0	>20	0	0	0	negative
173	m	<20	0	<20	0	0	>20	0	>20	0	0	0	negative
174	m	<20	0	<20	0	0	>20	0	>20	0	0	0	negative
175	m	<20	0	<20	0	0	>20	0	>20	0	0	0	negative
176	m	<20	0	<20	0	0	>20	0	>20	0	0	0	negative
213	f	<20	0	<20	0	0	>20	0	>20	0	0	0	negative
214	f	<20	0	<20	0	0	>20	0	>20	0	0	0	negative
215	f	<20	0	<20	0	0	>20	0	>20	0	0	0	negative
216	f	<20	0	<20	0	0	>20	0	>20	0	0	0	negative

The results presented in table 13 were summarized in figure 30 and figure 31

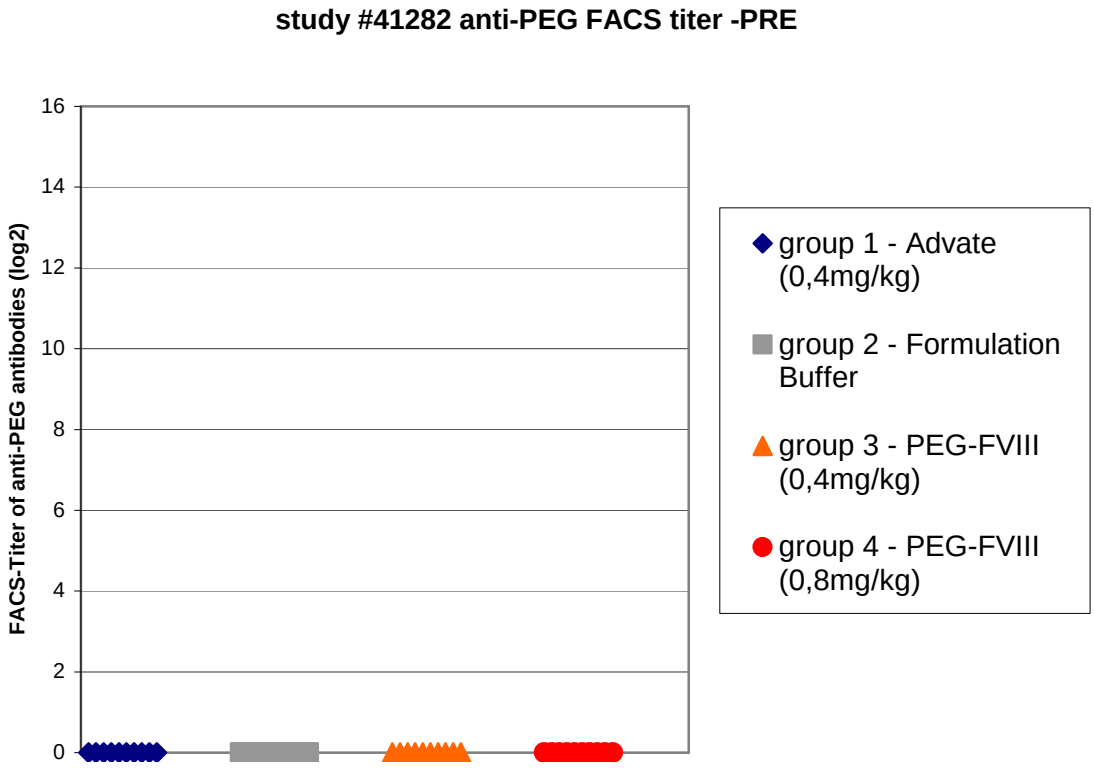

Figure 31: Diagram of anti-PEG FACS titers from rat plasma samples taken before immunization

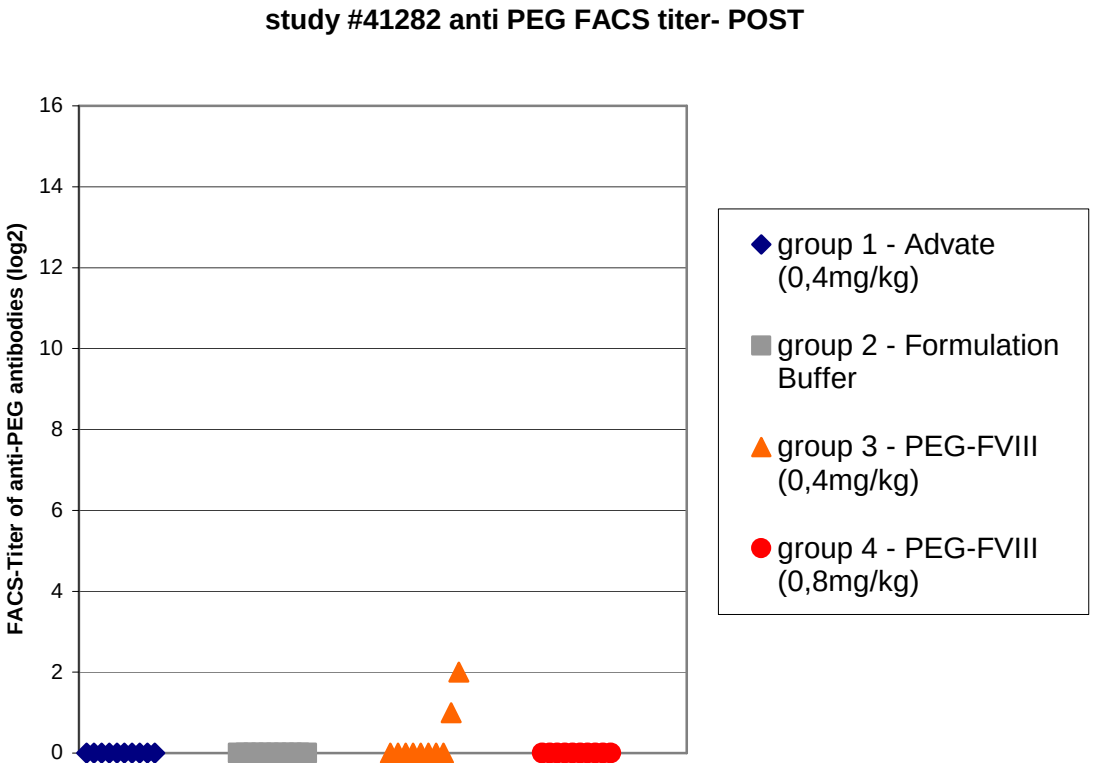

Figure 32: Diagram of anti-PEG FACS titers from rat plasma samples taken after immunization

4.4 Anti-PEG antibodies in healthy human donors

The aim of this study was to evaluate the incidence of anti-PEG antibodies within a healthy human population. This study in healthy blood donors provides important information for the screening of patients to be included in clinical studies of the new drug candidates. In figure 33 you can see an example of a anti-PEG IgG positive plasma sample with a MFI of 46.6. After pre-incubation with soluble PEG, the MFI goes down to 10.7.

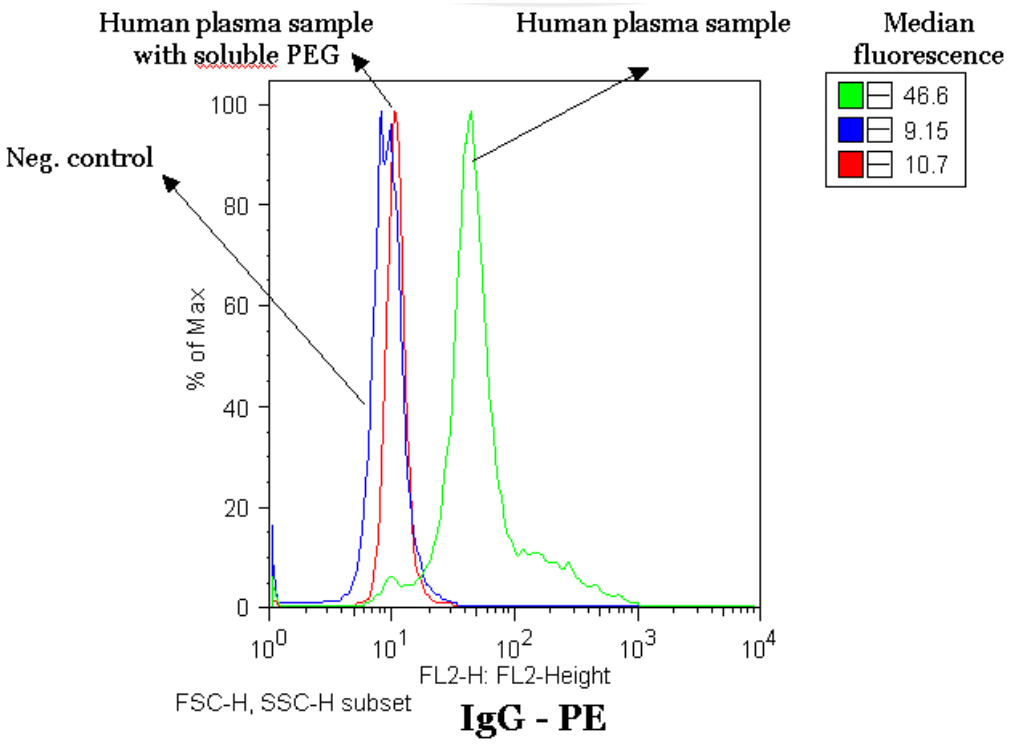

Figure 33: Example of a IgG anti-PEG positive human plasma sample The soluble PEG was given in a 5% concentration of total volume.

Table 14: Screening of human plasma samples from healthy donors. Samples were analyzed for IgG and IgM anti-PEG antibodies

Human Plasma Sample - Analyses

492 samples of healthy human plasma donors

positive samples	94	19,10%
IgG positive only	30	6,10%
IgM positive only	55	11,18%
IgG/IgM positive	9	1,83%

5. Discussion

A number of protein therapeutics have been successfully conjugated with polyethylene glycol (PEG) in order to increase the serum half-life and to reduce immunogenicity of these proteins. It was shown in recent reports that treatment with PEGylated protein therapeutics leads to the development of anti-PEG antibodies in animal disease models and patients. These antibodies caused a rapid clearance of the PEGylated proteins from the circulation (Armstrong et al 2006). Moreover, a considerable amount of anti-PEG antibodies in healthy donors was reported in contrast to previous studies (Armstrong et al 2003).

The findings of anti-PEG antibodies raise the question whether

1. the presence of anti-PEG antibodies may alter the pharmacokinetic profile of the PEGylated conjugate and eventually render treatment less effective or ineffective, necessitating a modification of dosing recommendations;
2. PEGylated coagulation factors induce anti-PEG antibodies leading to a less effective treatment as mentioned in patients with hemophilia.

The objective of my project was to establish an assay for the detection of anti-PEG antibodies. This assay should be suitable for monitoring the development of anti-PEG antibodies in animal disease models, in healthy human plasma donors and finally in patients treated with PEGylated proteins. The study in healthy blood donors should provide important information for the screening of patients to be included in clinical studies of the new PEGylated drug candidates.

Based on the results obtained in my project we can conclude that the flow cytometric approach to detect anti-PEG antibodies in plasma is a suitable approach that meets the requirements of accuracy, reproducibility and specificity. Moreover the new assay represents a significant improvement in comparison to earlier detection methods (Fisher et al).

5.1 Anti-PEG antibody detection assay

After the initial experimental set up was established, several studies had to be done in order to meet the requirements for the test:

- All antibodies to be used had to be titrated to find out the best concentration that would give an optimal signal to noise ratio. A combination of an 1:32 dilution of the primary antibody together with a dilution of 1:32 of the secondary antibody provided the optimal signal to noise ratio.

- The optimal concentration of the TentaGel beads had to be found. Our results after changing the concentration of the beads showed no difference in median fluorescence intensity (MFI) at all.

- In the early phase of developing the assay we decided to modify the TentaGel beads by adding a branched PEG reagent to the functional group of the linear PEGs that are grafted on the surface of the beads. This branched PEG is the same that Baxter Innovations GmbH is currently using to modify FVIII or VWF in order to increase the half life of Factor VIII. The additional PEGylated beads have the advantage of covering all potential epitopes that maybe present within the PEGylated protein therapeutics. After comparing both type of beads, the results show the median fluorescence intensities of the assay run with additional PEGylation of the beads, were the same or even higher than the run without additional PEGylation. The anti-PEG antibody detection assay with the additional PEGylated beads has an increased sensitivity, which could be to the enlargement of the PEG-surface on the beads through the branched PEG. Moreover, the additional PEGylation of the beads has some more advantages. First of all the beads seem to be less auto fluorescence. This result was seen in almost every experiment where we compared both type of beads with each other. Another improvement we noticed is an easier handling of our modified TentaGel beads due to almost no aggregation.

- We could show that after coupling the TentaGel beads with a branched PEG reagent, the stability of the beads was maintained. However, to ensure that the beads of the same lot would be stable during a whole preclinical or clinical study, we decided to lyophilize the TentaGel beads after the PEGylation reaction and test the lyophilized beads for stability under different conditions. Our results demonstrate, that the PEGylated beads are stable for at least 4 weeks.

- An important issue was to proof the specificity of the test. For this purpose we did a competition assay. We observed a reduction of the MFI after pre-incubation with the competitor and an increase again after diluting the competitor. Based on these results, we conclude that the detected antibodies in our assay were specifically directed against PEG.

- To monitor the potential development of anti-PEG antibodies in disease models it was necessary to define a cut off to be able to distinguish between a positive and a negative sample. For this purpose, blood was taken from untreated mice or rats and the plasma was screened using the anti-PEG antibody detection assay. Non of the animals tested showed a MFI value above background., It seems that there are no natural anti-PEG antibodies in mice or rats, which is exactly the result we would expect, due to the fact that they have never been confronted with PEG in their life. The results were taken to define the cut off using the 95% percentile, which means that we include in our assay 5% of false positive.

- A validation study had to be done to comply with quality requirements that were necessary to monitor potential development of anti-PEG antibodies in rat plasma within a study under Good Laboratory Practice (GLP) conditions. For this purpose, inter- and intra-assay variation, specificity and linearity were assessed to proof the reliability and accuracy of the method. We could proof the accuracy, reproducibility and specificity of the anti-PEG detection assay. Therefore the test fulfilled all requirements to work under GLP conditions.

5.2 Anti-PEG antibodies in mouse

In the course of the factor VIII Half Life project two proteins were PEGylated to elongate the half life of FVIII: rFVIII and rVWF, the carrier molecule of FVIII. Two studies were done in mice to compare the immunogenicity of rVWF and PEGylated rVWF. The potential development of anti-PEG antibodies was monitored.

As our results from study 1 show, no animal from a group that was treated with proteins without PEGylation showed an immune reaction. On the other hand we could detect anti-PEG antibodies in some animals in both groups that were treated with PEGylated proteins. From these results we conclude, that IgM and IgG anti-PEG antibodies develop after treatment with PEGylated proteins.

Study 2 had a similar study design as study 1, but there were major differences in the results: not only groups that were treated with PEGylated proteins showed anti-PEG antibodies. Even animals that just received formulation buffer showed a log 2 titer of 3. On the other hand only animals that were treated with PEGylated proteins developed IgG anti-PEG antibodies. One possible explanation for the results we obtained in study 2 may be a general, unspecific immune reaction of the animals due to an viral or bacterial infection.

5.3 Anti-PEG antibodies in rat

The aim of the 30-days intravenous toxicity study in rats was to evaluate the potential toxicity of a PEGylated recombinant FVIII administered intravenously in rats every other day for 30 days (15 doses). This study was conducted in accordance with FDA Regulations for Good Laboratory Practice (GLP). We had 4 different groups with different target doses. Blood was taken before and after treatment and the plasma was analyzed for anti-PEG antibodies. It can be seen that only 2 animal showed a titer of anti-PEG antibodies after treatment with PEGylated protein, and only one from these animals was counted as positive, because the difference

between log 2 titer values from pre-immunization and from post-immunization was ≥ 2. We could not see any development of anti-PEG antibodies within this study in rats. We conclude from our results that most of the animals were becoming tolerant for PEG after receiving repeated high doses of PEG within one month.

5.4 Anti-PEG antibodies in humans

The aim of this study was to evaluate the incidence of anti-PEG antibodies within a healthy human population. This study in healthy blood donors provide important information for the screening of patients to be included in clinical studies of the new drug candidates. Our study confirmed former results that pre-formed anti-PEG antibodies are occurring more often then expected, related to studies in the 1980s (Armstrong et al, ASH 2003) and present a major concern for the development of any PEGylated protein therapeutic, that should be considered. Nowadays PEG is widespread in a lot of different products, starting from make-up and food to therapeutics. Whether those antibodies that we detected in healthy human plasma donors are natural occurring antibodies or are pre-formed because the human individuals were confronted with PEG all their live, is yet unclear. To answer this question I suggest To analyze the B-cell receptor for somatic mutations. This would be a proof for affinity maturation by somatic hypermutations.

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5.5 Overall conclusion

We established a flow cytometric anti-PEG antibody detection assay that is accurate, reliable, reproducible and specific. Therefore, the assay fully complies with quality requirements that are necessary to run the test under FDA Regulations for Good Laboratory Practice (GLP). In addition we could show that the modification of the TentaGel beads provided a major improvement of the anti-PEG antibody detection assay.

Our results within the animal disease models indicate, that there is a potential to develop anti-PEG antibodies after treatment with PEGylated protein therapeutics. We did not see an immune reaction to PEGylated rFVIII. However, we could see antibody developing in animal disease models when treated with PEGylated rVWF. Maybe some epitopes get covered when PEG is attached to FVIII and therefore no immune reaction could be seen. Or the PEGylation of rVWF forms new epitopes and cause antibody developing. Those antibodies may recognize epitopes as well that we are presenting within our assay. Another possible explanation could be the different treatment schedules used in the studies. The study in rats with the PEGylated rFVIII used very high and repeated doses within one month. Therefore it is likely that the animals were becoming tolerant for PEG.

Our study with healthy human plasma donors confirm prior results that natural anti-PEG antibodies are occurring more often then expected, relating to studies in the 1980s (Armstrong et al, ASH 2003) and present a major concern for any PEGylated protein therapeutic development, that should be considered. We do not know whether the antibodies that we detected in healthy human plasma donors were natural occurring antibodies or pre-formed antibodies due to the fact that human individuals were confronted all their live with PEG.

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8. Abbreviations

PEG	Polyethylen Glycol
FACS	Fluorescence Activated Cell Sorting
FVIII	blood coagulation factor VIII
VWF	von Willebrand Factor
IgG	Immunglobulin G
IgM	Immunglobulin M
PE	Phycoerythrin
APC	Allophycocyanin
DPBS	Dulbeccos phosphate buffered saline
NaCl	Sodium Chloride
HCl	Hydrochloric acid
WFI	Water For Injection
MHC	Major Histocompatibility Complex
HLA	Human leukocyte antigen
MFI	Median Fluorescence Intensity
FDA	Food and Drug Administration
GLP	Good Laboratory Practice