The present invention relates to an anti-factor IX activating catalytic antibody and to a pharmaceutical composition comprising said activating catalytic antibody. The present invention also relates to a method of prognosing the outcome of acquired hemophilia and of treatment of acquired hemophilia, hemophilia A, B and C.
Method of prognosing the outcome of acquired hemophilia and of treatment of hemophilia.

The present invention relates to an anti-factor IX activating catalytic antibody and to a pharmaceutical composition comprising said activating catalytic antibody. The present invention also relates to a method of prognosing the outcome of acquired hemophilia and of treatment of acquired hemophilia, hemophilia A, hemophilia B and hemophilia C.

Coagulation which is a mechanism essential for animal survival comprises a cascade of enzymatic reactions involving coagulation factors. Coagulation may be induce through two pathways, contact activator pathway and tissue factor pathway in which the coagulation factors are differently implicated (FIG. 1). Each coagulation factor exists in an inactive precursor form and in an activated form. In their activated forms factors II, VII, IX, XI and XII act as serine protease. In the final step of the coagulation cascade, soluble fibrinogen is changed into fibrin filaments which surround the circulating cells.


Hemophilia A is a X-chromosome-linked disorder causing serious and frequent blood coagulation problem. This disease is characterized by the absence of factor VIII or of a functional factor VIII. Hemorrhages in patients with hemophilia
A can be treated with factor VIII. This therapy has limitations such as the short half life of factor VIII and the administration by intravenous infusion. Furthermore, approximately 10 to 30% of hemophilia A patients develop inhibitory antibodies against factor VIII, also referred to as factor VIII inhibitors, leading to treatment complications (Penner et al. (2001) Haemophilia 7:1 03-8). These factor VIII inhibitors neutralize factor VIII pro-coagulant activity by reducing the levels of circulating factor VIII (Kessler et al. (2000) Haematologica 85:57-61) and sterically preventing the interaction of factor VIII with activated factor IX, von Willebrand factor, phospholipids, thrombin and factor X.

Hemophilia B is a partial or complete deficiency of factor IX. Hemophilia B is a sex-linked recessive disorder with clinical symptoms including hemorrhages and hemarthrosis. This disorder is not as common as hemophilia A, occurring in 1 in 100,000 males. Inhibitory antibodies against factor IX have been reported in patients with hemophilia B with an occurrence of more than 1% of patients as a consequence of treatment. One of these antibodies was characterized as binding to factor IX by recognizing the peptide 175-182 of factor IX which comprises the cleavage site for this factor (180-181), thereby preventing factor IX from fulfilling its function in the cascade of coagulation (Takahashi et al. (1994) British Journal of Haematology 88:166-173).

Hemophilia C is a hereditary bleeding disorder caused by a deficiency of factor XI. Hemophilia C is an autosomal recessive disorder with clinical symptoms including excessive bleeding during surgery, delivery or menstruation. Its incidence is 1 in 100,000.

Acquired hemophilia is a related disease; it is a severe hemorrhagic autoimmune disorder that occurs in about one per one million individuals each year. The disorder is characterized by the spontaneous development of auto-antibodies directed against endogenous factor VIII. In most patients, anti-factor VIII autoantibodies are idiopathic. IgG from some patients with acquired hemophilia are also able to hydrolyze factor VIII (Wootia et al. (2008) J Immunol 180:7714-20). Acquired hemophilia occurs mostly during the post-partum period, in autoimmune diseases, during the development of malignancies and as side effects to drug administration (Delgado et al. (2003) Br J Haematol 121:21-35). Clinical features include bleeding in mucosal and soft tissues, hematuria,
hematemesis or melaena and prolonged post-partum or post-operative bleeding (Green et al. (1981) Thromb Haemost 45 (3):200-3). The reported mortality in one year following diagnosis is between 6.2 and 44.3 percent (Collins et al. (2007) Blood 109 :1870-7).

Due to the complex etiology of acquired hemophilia, no clinical parameter is known to predict the outcome of acquired hemophilia. There is thus a need to find parameters which allow the prognosis of the outcome of the disease. There is, thus, also a need to find new methods of treatment for hemophilia patients who have developed an immune response against factor VIII and for patients with acquired hemophilia.

In this study, the inventors identified the presence of catalytic antibodies directed against factor IX in some patients with acquired hemophilia. These antibodies have the ability to hydrolyze and activate factor IX. The inventors also observed a correlation between the presence of these antibodies in the patients and a better survival rate, one year after the disease was diagnosed. On the basis of these results, it is submitted that such antibodies could be used for the therapy of patients with blood coagulation disorders, in particular with acquired hemophilia, hemophilia A, B and C.


It was also previously reported that some murine IgG monoclonal antibodies directed against factor IX could mimic factor VIII to some extent and enhanced the catalytic activity of factor IX on factor X, without altering the affinity of factor X for factor IX. These anti-factor IX antibodies are not catalytic and act by inducing conformational changes in factor IX similar to those induced by factor VIII (Scheifflinger et al. (2008) J Thromb Haemost 6 :5-22, EP1 220923).

Thus for the first time the inventors have shown that catalytic antibodies against coagulation factor IX can be protective in the context of acquired hemophilia. Their results provide the basis for a prognosis of the outcome of acquired hemophilia. Their results also suggest a treatment for blood coagulation disorders in particular for patients with acquired hemophilia and hemophilia A, B and C based upon the administration of catalytic anti-factor IX antibodies.

**Antibody directed against coagulation factor IX which catalyzes proteolysis of coagulation factor IX and yields activated factor IX.**

The present invention relates to an antibody directed against coagulation factor IX, wherein said antibody catalyzes proteolysis of coagulation factor IX and yields activated coagulation factor IX.

The terms “factor VIII, IX, X or XI” or "coagulation factor VIII, IX, X or XI" have the same meaning and refer to factors which are implicated in the coagulation cascade as shown in FIG. 1.
The amino acid sequence of circulating human factor IX is shown in SEQ ID NO: 1.

As used herein "activated factor IX" or "factor IXα" refers to a cleaved, activated form of factor IX which is able to process and activate factor X. In the coagulation cascade factor IX is activated by factor Xα by cleaving the bonds between Arg^{145}- Ala^{146} and Arg^{190}- Val^{191} of SEQ ID NO: 1.

As used herein the terms "antibody" and "immunoglobulin" have the same meaning and are used indifferently in the present invention. Antibody refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which immunospecifically binds an antigen. As such, the term antibody encompasses not only whole antibody molecules, but also antibody fragments as well as variants of antibodies, including derivatives such as humanized antibodies. In natural antibodies, two heavy chains are linked to each other by disulfide bonds and each heavy chain is linked to a light chain by a disulfide bond. There are two types of light chain, lambda (λ) and kappa (κ). There are five main heavy chain classes (or isotypes) which determine the functional activity of an antibody molecule: IgM, IgD, IgG, IgA and IgE. Each chain contains distinct sequence domains. The light chain includes two domains, a variable domain (VL) and a constant domain (CL). The heavy chain includes four domains, a variable domain (VH) and three constant domains (CH1, CH2 and CH3, collectively referred to as CH). The variable regions of both light (VL) and heavy (VH) chains determine binding recognition and specificity to the antigen. The constant region domains of the light (CL) and heavy (CH) chains confer important biological properties such as antibody chain association, secretion, trans-placental mobility, complement binding, and binding to Fc receptors (FcR), long half-life. The Fv fragment is the N-terminal part of the Fab fragment of an immunoglobulin and consists of the variable portions of one light chain and one heavy chain. The specificity of the antibody resides in the structural complementarity between the antibody combining site and the antigenic determinant. Antibody combining sites are made up of residues that are primarily from the hypervariable or complementarity determining regions (CDRs). Occasionally, residues from non hypervariable or framework regions (FR) influence the overall domain structure and hence the combining site.
Complementarity determining regions (CDRs) refer to amino acid sequences which, together, define the binding affinity and specificity of the natural Fv region of a native immunoglobulin binding-site. The light and heavy chains of an immunoglobulin each have three CDRs, designated L-CDR1, L-CDR2, L-CDR3 and H-CDR1, H-CDR2, H-CDR3, respectively. Therefore, an antigen-binding site includes six CDRs, comprising the CDR set from each of a heavy and a light chain V region. An idioype is the unique set of antigenic determinants (epitopes) of the variable portion of an antibody.

Framework Regions (FRs) refer to amino acid sequences interposed between CDRs, i.e. to those portions of immunoglobulin light and heavy chain variable regions that are relatively conserved among different immunoglobulins in a single species, as defined by Kabat et al. (Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, Md., 1991). As used herein, a "human framework region" is a framework region that is substantially identical (about 85%, or more, in particular 90%, 95%, or 100%) to the framework region of a naturally occurring human antibody.

The term "polyclonal antibody" as used herein refers to antibodies that are a mixture of immunoglobulin molecules secreted against one or more specific antigens. They can be derived from different B cell lines or be purified from the blood, serum or plasma of a mammal.

The term "monoclonal antibody" or "mAb" as used herein refers to an antibody molecule of a single amino acid composition, that is directed against a specific antigen and which may be produced by a single clone of B cells or hybridoma. Monoclonal antibodies may also be recombinant, i.e. produced by protein engineering.

The term "chimeric antibody" refers to an engineered antibody which comprises a VH domain and a VL domain of an antibody derived from a non-human animal, in association with a CH domain and a CL domain of another antibody, in particular a human antibody. As the non-human animal, any animal such as mouse, rat, hamster, rabbit or the like can be used. A chimeric antibody may also denote a multispecific antibody having specificity for at least two different antigens.
The term "humanized antibody" refers to antibodies in which the framework or "complementarity determining regions" (CDR) have been modified to comprise the CDR from a donor immunoglobulin of different specificity as compared to that of the parent immunoglobulin. In a preferred embodiment, a mouse CDR is grafted into the framework region of a human antibody to prepare the "humanized antibody".

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fv, Fab, F(ab')₂, Fab', dsFv, scFv, sc(Fv)₂, diabodies and multispecific antibodies formed from antibody fragments.

The term "Fab" denotes an antibody fragment having a molecular weight of about 50,000 and antigen binding activity, in which about a half of the N-terminal side of H chain and the entire L chain, among fragments obtained by treating IgG with a protease, papaine, are bound together through a disulfide bond.

The term "F(ab')₂" refers to an antibody fragment having a molecular weight of about 100,000 and antigen binding activity, which is slightly larger than the Fab bound via a disulfide bond of the hinge region, among fragments obtained by treating IgG with a protease, pepsin.

The term "Fab" refers to an antibody fragment having a molecular weight of about 50,000 and antigen binding activity, which is obtained by cutting a disulfide bond of the hinge region of the F(ab')₂.

A single chain Fv ("scFv") polypeptide is a covalently linked VH::VL heterodimer which is usually expressed from a gene fusion including VH and VL encoding genes linked by a peptide-encoding linker. The human scFv fragment of the invention includes CDRs that are held in appropriate conformation, preferably by using gene recombination techniques. "dsFv" is a VH::VL heterodimer stabilised by a disulphide bond. Divalent and multivalent antibody fragments can form either spontaneously by association of monovalent scFvs, or can be generated by coupling monovalent scFvs by a peptide linker, such as divalent sc(Fv)₂.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain.
(VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites.

The antibodies of the invention preferably are IgG with a long half-life. The antibodies according to the invention are catalytic and activate factor IX.

The term "catalytic antibody" also called "abzyme" or "proteolytic antibody" refers to an antibody capable of catalyzing the hydrolysis of a peptide bond. Catalytic antibodies include those antibodies with "endopeptidase" activity. "Endopeptidase" activity refers to the ability of an enzyme to catalyze the hydrolysis of at least one non-terminal peptide bond between two amino acid residues within a polypeptide of any length.

Preferably, the catalytic antibody of the invention is specific for factor IX. Specificity, as used herein, refers to the ability of a catalytic antibody to distinguish between the factor IX and any other polypeptides, based on their structural difference, such that the enzymatic action are upon the target protein to a reasonable degree unique.

Analyses of catalytic antibody components have shown that enzymatic activity often resides in the light chains, and antibody light chains isolated from multiple myeloma patients frequently demonstrate catalytic activity (Thiagarajan et al. (2000) Biochemistry 39:6459). Studies have provided evidence connecting some catalytic antibodies and serine proteases.

Catalytic antibodies of the invention may also include antibodies containing a catalytic active site representative of the serine protease family. Despite the diversity in primary amino acid sequence among individual members of the family, serine protease activity is supported by a highly conserved tertiary structure, which comprises a serine-histidine-aspartate triad. This triad has been observed in catalytic antibodies. For example, molecular modeling of the light chain of an antibody capable of hydrolyzing vasoactive intestinal polypeptide (VIP, a 28-amino acid neuropeptide) revealed an arrangement of Ser27a, His93, and Asp1 similar to the catalytic triad arrangement of a subfamily of serine proteases (Gao et al. J. Bio. Chem. 269: 32389-32393 (1994)). Studies have shown that the aspartate residue is not always essential for catalytic activity. The term "serine protease diad" as used herein, is the minimal structure of the catalytic site for a recombinant
catalytic polypeptide to maintain at least a portion of its catalytic activity. This structure comprises a histidine residue and a serine residue located within CDR1, CDR2, or CDR3 of an antibody VH or VL, where the residues are in a spatial relation to each other similar to their spatial alignment in a serine protease triad, such that the histidine can abstract the proton from the serine hydroxyl group, allowing the serine to act as a nucleophile and attack the carbonyl group of the amide bond within the protein substrate.

The antibodies according to the invention are capable of cleaving factor IX to an activated form which is, in turn, capable of cleaving and activating factor X. Preferably, activated factor IX has a molecular weight of 45 kDa. The terms "cleave" or "proteolyse" as used herein refer to the hydrolysis of at least one peptide bond within the amino acid chain of a peptide, a polypeptide or a protein.

In a preferred embodiment of the invention, the antibody of the invention proteolyses the sequence of human coagulation factor IX of SEQ ID NO: 1 between Arg\textsuperscript{145} and Ala\textsuperscript{146} and/or between Arg\textsuperscript{180} and Val\textsuperscript{181}.

Preferably, the antibody of the invention is a monoclonal antibody able to induce the cleavage of the bonds between Arg\textsuperscript{145} and Ala\textsuperscript{146} of SEQ ID NO: 1 and between Arg\textsuperscript{180} and Val\textsuperscript{181} of SEQ ID NO: 1.

The invention may also relate to a combination of at least two antibodies or polypeptides as defined above, wherein at least one antibody or polypeptide is able to cleave the bond between Arg\textsuperscript{145} and Ala\textsuperscript{146} of SEQ ID NO: 1 and at least one antibody or polypeptide is able to cleave the bond between Arg\textsuperscript{180} and Val\textsuperscript{181} of SEQ ID NO: 1.

Methods of production of antibodies

Antibodies according to invention may be produced by any technique known in the art, such as, without limitation, any chemical, biological, genetic or enzymatic technique, either alone or in combination.

For example an antibody according to the invention may be produced by standard techniques, e.g. immunisation to recover polyclonal antibodies or hybridoma production to product monoclonal antibodies, followed by a step of selection of the antibodies having the desired proteolytic activity.
In order to produce antisera containing antibodies according to the invention with the desired specificity, the factor IX can be used to immunize suitable animals, e.g., mice, rabbits, or primates. A standard adjuvant, such as Freund's adjuvant, can be used in accordance with a standard immunization protocol. The animal's immune response to the immunogen preparation may be monitored by taking test bleeds and determining the titer of reactivity to the antigen of interest. Further fractionation of the antisera to enrich antibodies specifically reactive to the antigen and purification of the antibodies can be accomplished subsequently, using methods well known from those skilled in the art.

The monoclonal antibodies may be made using the hybridoma method first described by Kohler et al. (1975) Nature 256:495.

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding 1986 Monoclonal Antibodies: Principles and Practice, pp.59-103, Academic Press).

Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which are substances that prevent the growth of HGPRT-deficient cells.

Preferred immortalized myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred are murine myeloma lines, such as those derived from MOPC-21 and MPC-I I mouse tumors available from the Salk Institute Cell Distribution
Center, San Diego, California USA, and SP-2 cells (and derivatives thereof, e.g., X63-Ag8-653) available from the American Type Culture Collection, Manassas, Virginia USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor (1984) J. Immunol 133:3001, Brodeur et al., Monoclonal Antibody Production Techniques and Applications, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA).

After hybridoma cells are identified that produce antibodies of the desired specificity the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding (1986) Monoclonal Antibodies: Principles and Practice, pp.59-103, Academic Press). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as tumors in a mammal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, salting-out, dialysis, filtration, concentration, fractional precipitation, ion-exchange chromatography, gel filtration chromatography, size-exclusion chromatography, adsorption chromatography, isoelectric focusing chromatography, hydrophobic chromatography, reversed phase chromatography, affinity chromatography such as Protein-A Sepharose, gel electrophoresis and isoelectric focusing gel electrophoresis which are used in combination, if necessary. Such methods are described for example in Baines et al. (1992) Methods in Molecular Biology 10:79-104, The Human Press, Inc). The purified antibody is then concentrated and dehydrated into liquid or solid to meet to its final use.

The capacity of an antibody to hydrolyze factor IX and yield activated factor IX may be readily assayed by the one skilled in the art using usual enzymatic test, such as described in example 3. For instance Factor IX can be labeled with a wide variety of labels known in the art, including hapten labels (e.g. biotin, or labels
used in conjunction with detectable antibodies such as horse radish peroxidase antibodies), mass tag labels (e.g. stable isotope labels), radioisotopic labels, metal chelate labels, luminescent labels (e.g. fluorescent, phosphorescent, and chemiluminescent labels, typically having quantum yield greater than 0.1). Electroactive labels etc. The antibodies to be tested can then be incubated with the labeled factor IX and the amount of hydrolysed factor IX is thereafter measured by any known methods depending on the type of labels which was used. For example, if factor IX was biotinylated, it is possible to use an immunoblot (kit BCIP/NBT) after migration on sodium dodecyl-sulfate polyacrylamide gel.

The factor IX proteolysing and activating activity of antibodies can also be evaluated by measuring the amount of activated factor X produced, for instance using a functional assay as described in example 3 below. To that end, the purified antibodies may be incubated with factor IX during a time sufficient for its cleavage and activation to occur. The mixture may be incubated with factor X, e.g. in the presence of phospholipid vesicles and CaCl₂. The formation of activated factor X is measured for example by assaying cleavage of a substrate for factor Xa. Advantage may be taken of the fact that activated factor X has amyldolytic activity against a synthetic substrate such as S2366.

In another method, the amount of activated factor X produced may be assessed in tests involving the formation of thrombin.

The antibodies of the invention can, for example, be produced by recombinant DNA techniques in a suitable expression system. The term "expression system" means a host cell and compatible vector under suitable conditions, e.g. for the expression of a protein coded for by foreign DNA carried by the vector and introduced to the host cell. Typically, a nucleic acid sequence encoding an antibody, in particular a monoclonal antibody, of the invention, or a fragment thereof, may be included in any suitable expression vector which may then be introduced into suitable eukaryotic or prokaryotic hosts that will express the desired antibodies.

The terms "vector", "cloning vector" and "expression vector" mean the vehicle by which a DNA or RNA sequence encoding the antibody can be introduced into a host cell, so as to transform the host and promote expression (e.g. transcription and translation) of the introduced sequence. An expression
vector is typically a plasmid, cosmid, episome, artificial chromosome, phage or a viral vector.


Other examples of vectors include replicating plasmids comprising an origin of replication, or integrative plasmids, such as for instance pUC, pcDNA, pBR, and the like.

Examples of viral vector include adenoviral, retroviral, herpes virus and AAV vectors. Such recombinant viruses may be produced by techniques known in the art, such as by transfecting packaging cells or by transient transfection with helper plasmids or viruses. Typical examples of virus packaging cells include PA317 cells, PsiCRIP cells, GPenv+ cells, 293 cells, etc. Detailed protocols for producing such replication-defective recombinant viruses may be found for instance in WO 95/1 4785, WO 96/22378, US 5,882,877, US 6,013,516, US 4,861,719, US 5,278,056 and WO 94/1 9478.

Host cells are transfected, infected or transformed by a nucleic acid and/or an appropriate vector as above described. The term "transformation" means the introduction of a "foreign" (i.e. extrinsic or extracellular) gene, DNA or RNA sequence to a host cell, so that the host cell will express the introduced gene or
sequence to produce a desired substance, typically a protein or enzyme coded by
the introduced gene or sequence. Transformed host cells are encompassed within
the scope of the invention.

Common expression systems include *E. coli* host cells and plasmid vectors,
insect host cells and Baculovirus vectors, and mammalian host cells and vectors.
Other examples of host cells include, without limitation, prokaryotic cells (such as
bacteria) and eukaryotic cells (such as yeast cells, mammalian cells, insect cells,
plant cells, etc.). Specific examples include *E. coli, Kluyveromyces* or
*Saccharomyces* yeasts, mammalian cell lines (*e.g.*, Vera cells, CHO cells, 3T3
cells, COS cells, etc.) as well as primary or established mammalian cell cultures
(*e.g.*, produced from lymphoblasts, fibroblasts, embryonic cells, epithelial cells,
nervous cells, adipocytes, etc.). Examples also include mouse SP2/0-Ag1.4 cell
(ATCC CRL1 581), mouse P3X63-Ag8.653 cell (ATCC CRL1 580), CHO cell in
which a dihydrofolate reductase gene (hereinafter referred to as "DHFR gene") is
defective (Urlaub G *et al.* (1980) *Proc Natl Acad Sci USA* 77(7):421-6-20), rat
YB2/3HL.P2.G1 1:6Ag.20 cell (ATCC CRL1 662, hereinafter referred to as "YB2/0
cell"), and the like. The YB2/0 cell is preferred, since ADCC activity of chimeric or
humanized antibodies is enhanced when expressed in this cell.

The Fab of the present invention can be obtained by treating an antibody
with a protease, papain. Also, the Fab can be produced by inserting DNA
encoding Fab of the antibody into a vector for prokaryotic expression system, or
for eukaryotic expression system, and introducing the vector into a procaryote or
eucaryote (as appropriate) to express the Fab.

The F(ab')2 of the present invention can be obtained treating an antibody
with a protease, pepsin. Also, the F(ab')2 can be produced by binding Fab'
described below via a thioether bond or a disulfide bond.

The Fab' of the present invention can be obtained treating F(ab')2 antigen
with a reducing agent, dithiotheritol. Also, the Fab' can be produced by inserting
DNA encoding Fab' fragment of the antibody into an expression vector for
prokaryote, or an expression vector for eukaryote, and introducing the vector into a
prokaryote or eukaryote (as appropriate) to perform its expression.

The scFv of the present invention can be produced by obtaining cDNA
encoding the VH and VL domains as previously described, constructing DNA
encoding scFv, inserting the DNA into an expression vector for prokaryote, or an expression vector for eukaryote, and then introducing the expression vector into a prokaryote or eukaryote (as appropriate) to express the scFv. To generate a humanized scFv fragment, a well known technology called CDR grafting may be used, which involves selecting the complementary determining regions (CDRs) from a donor scFv fragment, and grafting them onto a human scFv fragment framework of known three dimensional structure (see, e.g., W098/45322; WO 87/02671; US 5,859,205; US 5,585,089; US 4,816,567; EP 0173494).

Alternatively, catalytic antibodies can be produced as described in Barbas et al. ((1997) Science 28(5346):2085-92). The method provides a means to select antibody catalysts in vivo on the basis of their ability to carry out a chemical reaction.

Another alternative could be to produce anti-idiotypic antibodies against an antibody able to recognize a molecule able to cleave and activate factor IX, like factor XI. In fact, it was previously reported that anti-idiotypic antibodies could mimic proteolytic function of the parent antigen (Ponomarenko et al. (2007) Biochemistry 46(50):14598-609).

Therapeutic compositions

The antibodies of the invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

Where combined therapy is contemplated, pharmaceutical compositions may comprise the antibodies, fragments or immunoconjugates of the invention together with other molecules useful to treat a coagulation blood disorder, for example FVIII or FVIIa.

"Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.
The form of the pharmaceutical compositions, the route of administration, the dosage and the regimen naturally depend upon the condition to be treated, the severity of the illness, the age, weight, and sex of the patient, etc.

The pharmaceutical or therapeutic compositions of the invention can be formulated for a topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous or intraocular administration and the like.

Preferably, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

The doses used for the administration can be adapted as a function of various parameters, and in particular as a function of the mode of administration used, of the relevant pathology, or alternatively of the desired duration of treatment.

To prepare pharmaceutical compositions, an effective amount of the antibody may be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.
An antibody of the invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetables oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The preparation of more, or highly concentrated solutions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in
extremely rapid penetration, delivering high concentrations of the active agents to a small tumor area.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

The antibodies of the invention may be formulated within a therapeutic mixture to comprise about 0.0001 to 1.0 milligrams, or about 0.001 to 0.1 milligrams, or about 0.1 to 1.0 or even about 10 milligrams per dose or so. Multiple doses can also be administered.

In addition to the compounds formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; time release capsules; and any other form currently used.

In certain embodiments, the use of liposomes and/or nanoparticles is contemplated for the introduction of antibodies into host cells. The formation and use of liposomes and/or nanoparticles are known to those of skill in the art.

Nanocapsules can generally entrap compounds in a stable and reproducible way. To avoid side effects due to intracellular polymeric overloading,
such ultrafine particles (sized around 0.1 µιτ) are generally designed using polymers able to be degraded in vivo. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use in the present invention, and such particles may be easily made.

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs)). MLVs generally have diameters of from 25 nm to 4 µm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution in the core. The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations.

**Therapeutic applications**

The antibodies according to the invention and the medicament or pharmaceutical compositions comprising the antibody of the invention are intended to treat individuals with blood coagulation disorders.

As intended herein, the term "blood coagulation disorder" relates in particular to hemophilia, more particularly to acquired hemophilia, hemophilia A, hemophilia B and hemophilia C. More preferably, the blood coagulation disorder according to the invention is selected from the group consisting of acquired hemophilia and hemophilia A.

Hemophilia is a group of hereditary genetic disorders that impair the body's ability to control blood clotting or coagulation. In its most common forms, hemophilia A, the clotting factor VIII is absent partially or in totality. In hemophilia B, factor IX is partially or totally deficient, and in hemophilia C, factor XI is partially or totally deficient.

As intended herein, individuals with hemophilia A, B, or C may have been respectively treated by administration of factor VIII, factor IX or factor XI, and may have developed antibodies against these factors.

As used herein, "acquired hemophilia" relates to a severe hemorrhagic autoimmune disorder characterized by the spontaneous development of auto-antibodies directed against endogenous factor VIII. Clinical symptoms include
bleeding in mucosal and soft tissues, hematuria, hematemesis or melaena as well as prolonged post-partum or post-operative bleeding.

In the context of the invention, the term "treating" or "treatment" means reversing, alleviating, inhibiting the progress of, or preventing the disorder or condition to which such term applies, or one or more symptoms of such disorder or condition. In particular, the treatment of the disorder may consist in promoting blood coagulation. Most preferably, such treatment leads to the complete restoration of blood coagulation.

According to the invention, the term "subject" or "individual" is intended for a human or non-human mammal (such as a rodent (mouse, rat), a canine, or a primate affected or likely to be affected by acquired hemophilia, hemophilia A, B or C. Preferably, the subject is a human.

Preferably, in particular where the individual is affected by hemophilia C, the individual according to the invention is at risk of excessive bleeding during surgery, delivery or menstruation. As intended herein "excessive bleeding" indicates that the individual may bleed more than a non-hemophilic individual in a same situation.

In a preferred embodiment the subject or individual is likely to receive the treatment as long as he presents detectable factor VIII activity. Preferably, his rate of expression of factor VIII is equivalent or higher than 1% or 2%, more preferably equivalent or higher than 3% of the rate of expression of factor VIII in a healthy subject. A "healthy subject" refers to a subject who has no coagulation disorders, more particularly who has no coagulation disorders which could modify his level of factor VIII. For example, in a healthy subject, the concentration of factor VIII in plasma is about 0.3 nM.

The term "therapeutically effective amount" is meant for a sufficient amount of antibody in order to treat acquired hemophilia, hemophilia A, B or C, at a reasonable benefit/risk ratio applicable to any medical treatment. It will be understood, however, that the total daily usage of the antibodies and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific antibody (ies)
employed; the specific composition employed, the age, body weight, general
health, sex and diet of the patient; the time of administration, route of
administration, and rate of excretion of the specific antibody employed; the
duration of the treatment; drugs used in combination or coincidental with the
specific polypeptide employed; and like factors well known in the medical arts. For
example, it is well known within the skill of the art to start doses of the compound
at levels lower than those required to achieve the desired therapeutic effect and to
gradually increase the dosage until the desired effect is achieved.

Method of prognosing the outcome of the disease in patients suffering of
acquired hemophilia

The inventors identified that patients with acquired hemophilia who have a
significantly higher level of factor-IX proteolysing and activating antibody have a
better survival rate than patients who have a lower level of factor-IX proteolysing
and activating antibody. Preferably, the factor-IX proteolysing and activating
antibody is an IgG.

Thus the present invention also relates to the use of an antibody as defined
above for the in vitro prognosis of the outcome of acquired hemophilia.

A method for assessing the chances of survival of acquired hemophilia
patients may be derived from these results, a high quantity of factor-IX
proteolysing and activating antibodies being indicative of a higher survival rate.

The invention thus also relates to a method of prognosing the outcome of
acquired hemophilia in an individual comprising the steps consisting of:

a) determining the level of antibodies directed against coagulation factor IX,

wherein said antibodies catalyze proteolysis of coagulation factor IX and yield
activated factor IX, or rate of conversion of coagulation factor IX into activated
factor IX, in a biological sample of said individual;

b) comparing said level or rate to at least one predetermined value; and

c) predicting the outcome of the disease based upon the comparison of said

level or rate with the predetermined value.

Quantitation of the level of factor IX proteolysing and activating antibodies,
in particular IgG, present in a patient with acquired hemophilia can be achieved by
using known methods. It can involve purifying antibodies from a patient's sample
by standard purification techniques (e.g. affinity chromatography with Protein A, Protein G, anti-immunoglobulin, or the antigen itself). As mentioned above, factor IX can be labeled with a wide variety of labels known in the art, including hapten labels (e.g. biotin, or labels used in conjunction with detectable antibodies such as horse radish peroxidase antibodies), mass tag labels (e.g. stable isotope labels), radioisotopic labels, metal chelate labels, luminescent labels (e.g. fluorescent, phosphorescent, and chemiluminescent labels, typically having quantum yield greater than 0.1), electroactive labels etc. The biological sample, or antibodies purified therefrom can then be incubated with the labeled factor IX and the amount of hydrolysed factor IX is thereafter measured by any known methods depending on the type of labels which was used. For example, if factor IX was biotinylated, it is possible to use an immunoblot (kit BCIP/NBT).

The factor IX proteolysing and activating activity of antibodies, in particular IgG, present in a patient with acquired hemophilia can be evaluated by measuring the amount of activated factor X produced or the rate of production. For instance, the biological sample or antibodies purified therefrom may be incubated with factor IX during a time sufficient for its cleavage and activation to occur. The mixture may then be incubated with factor X, e.g. in the presence of phospholipid vesicles and CaCl₂. The formation of activated factor X is measured for example by assaying cleavage of a substrate for factor Xa. Advantage may be taken of the fact that activated factor X has amydolytic activity against a synthetic substrate such as S2366.

In another method, the amount of activated factor X produced or the rate of production may be assessed in tests involving the formation of thrombin.

As used herein the "concentration of" or "amount of" or "level of" factor-IX proteolysing and activating IgG refers to the number of such IgG in the biological sample.

The "rate of conversion of coagulation factor IX into activated factor IX" refers to the level of factor IX activation mediated by anti-factor IX catalytic antibodies in function of time. This rate can, preferably, be measured in mmol of activated factor IX/min/mol of total IgG.

The rate of conversion of coagulation factor IX into activated factor IX may be measured using the functional assay as described in example 3 below.
The predetermined value can be a single value such as a level of factor-IX proteolysing and activating IgG or a rate of conversion of coagulation factor IX into activated factor IX. Said single value may be for instance a mean level of factor IX-proteolysing and activating IgG or a mean rate of conversion of coagulation factor IX into activated factor IX. A level of factor IX-proteolysing and activating IgG or a rate of conversion of coagulation factor IX into activated factor IX lower than the predetermined value is indicative of a prognosing of low survival. In particular, the level of factor IX-proteolysing and activating IgG or of the rate of conversion of coagulation factor IX into activated factor IX measured in the biological sample of the individual may be at least 10%, 20%, 30%, 40%, 50%, 60%; 70%, 80%, 90%, 100%, 150% or 200% lower than the predetermined value.

In a preferred embodiment, the predetermined value corresponds to a null level and the absence of antibodies directed against coagulation factor IX value is indicative of a low probability of survival within 1 year.

In the method of the invention, the predetermined value may also comprise multiple ranges of level of factor IX-proteolysing and activating IgG measured or of rate of conversion of coagulation factor IX into activated factor IX in a population of individuals with acquired hemophilia, said population being divided into quantiles according to the concentration of factor IX-proteolysing and activating IgG or according to the rate of conversion of coagulation factor IX into activated factor IX, the lowest quantiles being individuals with the lowest chances of survival at one year after the diagnosis. For instance, the population of individuals with acquired hemophilia could be divided into 2, 3, 4, or 5 quantiles. Individuals in the lowest quantile, or of the two lowest quantiles (if the population is divided e.g. into 4 or 5 quantiles) would be considered as individuals with the lowest chances of survival at one year after the diagnosis.

Alternatively, the predetermined value can be the therapeutically effective amount of antibodies directed against factor IX as defined above which is necessary to treat a subject with acquired hemophilia or hemophilia A, B or C, optionally with factor VIII inhibitors.

In another embodiment, the predetermined value of activation of factor IX is 0.15 mmol/min/mol, preferably 0.187 mmol/min/mol, more preferably 0.2 mmol/min/mol and still preferably 0.25 mmol/min/mol. The patients with a rate of
activation of factor IX higher than this predetermined value having increased chances of survival compared to patients with a lower rate, one year after diagnosis.

Accordingly, the method of the invention may further comprise monitoring the level of antibodies directed against coagulation factor IX or the rate of conversion of coagulation factor IX into activated factor IX in the course of time, wherein an increase of the level of antibodies directed against coagulation factor IX or of the rate of conversion of coagulation factor IX into activated factor IX in the course of time is indicative of a higher probability of survival within 1 year. The monitoring is typically performed by determining the level of antibodies directed against coagulation factor IX or the rate of conversion of coagulation factor IX into activated factor IX at different points in time, for instance of 2 weeks, 1 month, 2 months, 3 months etc.

According to the invention, the biological sample may be a blood sample, a plasma sample or a serum sample.

The invention will be further illustrated in view of the following figures and examples.

**FIGURES**

**FIG. 1** depicts the schematic representation of the coagulation cascade.

**FIG. 2** depicts a Western blot of the proteolysis profile of factor VIII after 24 hours of incubation with IgG from three different patients (1, 8, and 16) compared to the hydrolysis profile of control IgG from IVIg (IVIg, Intravenous Immunoglobulin) and to the proteolysis profile of factor VIII alone after 0 or 24 hours of incubation.

**FIG. 3** depicts on a Western blot the proteolysis profile of factor IX after 24 hours of incubation with IgG from three different patients (1, 8, and 16) compared to the hydrolysis profile of normal IgG from IVIg (IVIg) and of factor VIII alone after 0 or 24 hours of incubation.

**FIG. 4** depicts the rate of IgG-mediated proteolysis of factor IX (in mmoles of factor IX/min/moles of IgG, vertically) as a function of the binding of IgG of 65 patients to factor IX as tested by enzyme-linked immuno assay against recombinant factor IX (horizontally).
FIG. 5 depicts the rate of IgG-mediated proteolysis of factor IX (in mmoles of factor IX/ min/moles of IgG, vertically) as a function of the rate of IgG-mediated proteolysis of factor VIII (in mmoles of factor VIII/min/moles of IgG, horizontally) for 45 patients.

FIG. 6 depicts the average proteolysis, as evaluated from three independent experiments, of labeled factor IX by IgG from patient 16 (in nM of FIX/min, vertically) in the presence of increasing concentrations of unlabelled factor IX (in µM, horizontally). Inset depicts the reciprocal of the substrate concentration versus that of the velocity (r=0.99) for the five highest concentrations of unlabelled factor IX.

FIG. 7 depicts the cleavage of factor IX incubated in the presence of IgG from patient 16 and 32 (lanes 5 and 6) in comparison with human recombinant factor IX alone (lane 1), IgG purified from the plasma of patients 16 and 32 alone (lanes 2 and 3), and human recombinant activated factor IX (lane 4) as observed on a 4 to 12 percent sodium dodecyl-sulfate polyacrylamide gel electrophoresis and after coloration of the proteins by colloidal Coomassie blue.

FIG. 8 depicts the activation of factor IX (in mmoles of factor IX/min/moles of IgG) after incubation with IgG from 65 patients with acquired hemophilia in comparison with the activation of factor IX by incubation with control IgG from IVIg (IVIg), with IgG from a patient with congenital hemophilia B (HJC) or with factor IX incubated alone (FIX).

FIG. 9 depicts the rate of IgG-mediated proteolysis of factor IX (in mmoles of factor IX/min/moles of IgG, vertically) plotted as a function of the rate of IgG-mediated activation of factor IX (in mmoles of factor IX/min/moles of IgG, horizontally).

FIG. 10 depicts the generation of thrombin (in nM, vertically) in factor VIII-deficient plasma devoid of platelets supplemented with activated factor IX and factor VIII at 0, 3, 10 or 30% of the level found in normal plasma (0%, 3% FVIII, 10% FVIII, 30% FVIII) in function of time (min, horizontally). The results are representative of two independent experiments.

FIG. 11 depicts the association between the survival of 54 patients one year following diagnosis of acquired hemophilia and either sex, age, cancer as a pre-
existing condition, FVIII activity, or rate of IgG-mediated activation of factor IX (vertically) in function of time (days, horizontally).

**EXAMPLES**

**EXAMPLE 1: Patients included in the study**

Frozen plasma samples from 65 patients with acquired hemophilia were obtained at the time of diagnosis from CHU de Rouen (Etude Sacha: 41 patients), CHU de Caen (11 patients), Hopital Cochin (Paris), Hopitaux du Kremlin-Bicetre (Bicetre), Nimes and CHU de Compiegne (France: 13 patients), in accordance with the local ethical regulation. 'Etude Sacha' includes clinical data from 82 patients with acquired hemophilia A collected in France. All 'Sacha' patients with available plasma samples at the time of diagnosis (n=41) were enrolled in our ancillary study. Criteria for inclusion of the patients were a residual factor VIII activity below 30 percent (FVIII above 30 percent in the case of 2 patients), an inhibitory titer above or equal to 1 Bethesda units (BU)/ml, a prolonged activated partial thrombosplatin time (aPTT) and normal levels of other factors of the intrinsic pathway and of von Willebrand factor.

**EXAMPLE 2: Determination of the catalytic activity of IgG against factors VIII and IX**

**METHODS**

**Determination of factor VIII-inhibitory activity**

Factor VIII-inhibitory activity was measured in plasma using the modified Bethesda assay (Kasper et al. (1975) *Thromb Diath Haemorrh* **34**:869-72). Plasma was heated 1 hr at 56°C. Heated plasma was incubated with an equal volume of pooled citrated human plasma (Dade-Behring, Marburg, Germany) for 2 hours at 37°C. Residual factor VIII activity was measured in a 1-stage clotting assay, as described. The detection limit of the assay was 0.3 BU. One BU (Bethesda units) corresponds to the inverse of the dilution of plasma that yields 50 percent residual factor VIII activity.
Purification of IgG

IgG was isolated from plasma by affinity-chromatography on protein G Sepharose (Amersham Pharmacia Biotech, Buckinghamshire, England). Intravenous immune globulin (Sandoglobulin®, CSL-Behring, Bern, Switzerland) was used as a source of normal IgG. To exclude potentially contaminating proteases, size-exclusion chromatography of patients’ IgG and IVIg (Intravenous Immunoglobulin) was performed on a superose-12 column (Amersham) equilibrated with 50 mM Tris, 8 M urea and 0.02 percent NaN₃ pH 7.7, at a flow rate of 250 µl/min. IgG-containing fractions were pooled and dialyzed against PBS-0.01 percent NaN₃ for 48 hr at 4°C, followed by dialysis against 50 mM Tris (pH 7.7), 100 mM glycine, 0.02 percent NaN₃, 5 mM CaCl₂ (catalytic buffer) for 24 hr at 4°C. We have previously demonstrated that urea-treated purified IgG retain the inhibitory activity towards factor VIII (Lacroix-Desmazes et al. (2002) N Engl J Med 346:662-7). The purity of IgG preparations was assessed by Western blotting under non-reducing conditions and by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) analysis of trypsin digests of the IgG preparations. IgG was quantified by optical density measurements at 280 nm.

Proteolysis of biotinylated factors

Human recombinant factor IX (BeneFIX®) and factor VIII (Kogenate® FS or Helixate®) were used in all experiments. Recombinant human factor IX and VIII were dialyzed against 100 mM borate (pH 7.0), 150 mM NaCl, 5 mM CaCl₂ (Borate buffer) and reacted with sulfo-NHS-LC-biotin (Pierce) for 2 hr at 4°C. Biotinylated factor IX and VIII were dialyzed against catalytic buffer for 3 hr at 4°C, aliquoted and stored at -20°C until use. Biotinylated factors (185 nM) were incubated in catalytic buffer with IgG (10 µg/ml, 67 nM) for 24 hr at 37°C. Following incubation for 24 hours at 37°C, digestion profiles were analyzed by mixing samples mixed with Laemmli’s buffer without β-mercaptoethanol (1:1 V/V), and subjecting 20 µl of each sample to 10 percent sodium dodecyl-sulfate polyacrylamide gel electrophoresis. Protein fragments were then transferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel, FRG). Following overnight blocking in TBS-0.2 percent tween 20 at 4°C, membranes were incubated with streptavidin-coupled alkaline phosphatase (KPL, Gaithersburg, MD, USA) diluted
1:4000 in blocking buffer, for 60 min at room temperature. After washing in TBS-
0.1 percent tween 20, labeled proteins were revealed using the BCIP/NBT kit.

**Calculation of rates of proteolysis from immunoblots.**

High-resolution images were acquired by scanning the immunoblots using a SnapScan 600 scanner (Agfa, Mortsel, Belgium). Black and white images were converted to negatives using the Adobe Photoshop CS2 (Version 9.0.2) software. A macro was written using the NIH image 1.62b7 software (OD macro©, Heudes D & Nicoletti A, CRC, INSERM, France) in order to calculate mean image densities. Briefly, the negative images were imported into the NIH image 1.62b7 software using the OD macro®. The negative images were converted back to the positive mode by applying an arithmetic logarithmic (log) process. The "log process" does not affect the image pixels. For calculating the rates of factor IX proteolysis, we measured, for each sample, the mean density i) of the total area of the lane of the factor IX migration profile and ii) of the area of the protein bands with molecular weights below 50 kDa. The percentage of factor IX proteolyzed was calculated as the ratio of the mean density of the hydrolyzed area over the mean density of the total area of the lane. Spontaneous hydrolysis occurring upon incubation of factor IX in the presence of buffer alone was considered as the background level and was subtracted from each analysis. Data were expressed as mmol of factor IX proteolyzed per minute per mol of IgG. Significant differences between the rates of factor IX proteolysis of patients' IgG and that of immune globulin were assessed using an analysis of variance post-hoc test (Fisher's PLSD). The reported P values are one-sided. The method was essentially similar for the calculation of IgG-mediated factor VIII hydrolysis.

**Enzyme-linked immuno assay for anti-factor IX IgG.**

Plates (Nunc, Maxisorb) were coated with recombinant human factor IX (2 μg/ml, 1 hr) in phosphate-buffered saline (PBS, 10 mM sodium phosphate/0.14 M NaCl, pH 7.4) and blocked (1 hr) with PBS, BSA (1 percent, W/V). Purified IgG (0 to 67 nM, 50 μl) were incubated for 1 hr and the plates were washed with PBS-tween 20 (0.01 percent V/V). The plates were then incubated for 1 hr with peroxidase-linked mouse anti-human immunoglobulin antibodies (Jackson
Immu n o Research, Suffolk, UK). After washing with PBS-0.01 percent tween 20, the o-Phenylenediamine dihydrochloride (SIGMAFAST™ OPD, Lyon, France) substrate was added and the absorbance at 492 nm was measured using a UV-vis spectrophotometer (GENios, Tecan Trading AG, Switzerland). The optical density obtained in the case of wells lacking IgG was considered to represent the background level and was subtracted from the values obtained for each well incubated with purified IgG. ELISAs on different plates were standardized using the purified IgG from a hemophilia B patient who had developed anti-factor IX IgG following replacement therapy with exogenous factor IX (Patient HJC).

**Kinetic parameters for IgG-mediated factor IX proteolysis.**

Factor IX (800 nM) was labeled with $^{125}$I (Perkin-Elmer, USA) using iodo Gen (Pierce Chemical Co, Rockford, IL, USA) as described. The specific radioactivity was 0.9 µCi/µg. $^{125}$I-labeled factor IX (4.5 ng), was incubated for 24 hr at 37°C with the samples (25 pg/ml IgG and 0 to 55.8 µM of un-labelled FIX) in 40 µl of kinetic buffer (50 mM Tris, 150 mM NaCl, 5 mM CaCl$_2$, 0.1 percent (W/V) BSA, 0.1 percent (V/V) PEG). Samples were mixed 1:1 with Laemmli’s buffer without β-mercaptoethanol and were separated by SDS electrophoresis without being boiled; 25 µl of each sample was loaded per lane. Samples were separated by 7.5 percent SDS-PAGE in non-reducing conditions at room temperature in a mini-PROTEAN II system at 25 mA/gel, until the dye front reached the bottom of the gel. The gels were then dried and were exposed to autoradiography films (KODAK™ BioMax® MS-1 Autoradiography Film, Perkin-Elmer, USA). Autoradiographs were scanned to allow calculation of the rate of proteolysis of labeled factor IX. The data were fitted to the Michaelis-Menten equation by means of the DeltaGraph program (v5.5.2, Red Rock Software, Inc. Salt Lake City, UT).

**RESULTS**

Plasma from 65 patients was collected at the time of diagnosis of acquired hemophilia. The mean inhibitory activity against factor VIII in plasma ranged between 1 and 1050 BU/ml (Table 1).
Table 1. Characteristics of the patients at the time of diagnosis.

<table>
<thead>
<tr>
<th>Clinical cofactors</th>
<th>Nb of patients documented</th>
<th>Nb or % (mean ± SD)</th>
<th>% or (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex: male</td>
<td>65</td>
<td>40</td>
<td>66</td>
</tr>
<tr>
<td>Age (years)</td>
<td>54</td>
<td>68.1 ± 17.0</td>
<td>(25 - 92)</td>
</tr>
<tr>
<td>Preexisting conditions: cancer&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55</td>
<td>11</td>
<td>20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Standard biological cofactors</th>
<th>Nb of patients documented</th>
<th>Nb or % (mean ± SD)</th>
<th>% or (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor VIII activity in plasma (%)</td>
<td>51</td>
<td>6.0 ± 10.9</td>
<td>(0 - 59)</td>
</tr>
<tr>
<td>Inhibitory titer towards factor VIII (BU/ml)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>65</td>
<td>66.9 ± 148.6</td>
<td>(1 - 1050)</td>
</tr>
<tr>
<td>Factor IX activity in plasma (%)</td>
<td>18</td>
<td>117.5 ± 55.3</td>
<td>(64 - 268)</td>
</tr>
<tr>
<td>aPTT (measured/physiological value)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>37</td>
<td>2.4 ± 0.7</td>
<td>(1.3 - 4.2)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Other preexisting conditions were allergic drug reactions (n=5), autoimmune disorders (4), dermatologic disorders (3), diabetes (1), postpartum (2) and idiopathic (14).

<sup>b</sup>Inhibitory titers were assessed using the modified Bethesda assay.

<sup>c</sup>aPTT: activated partial thromboplastin time.

As reported, IgG from some patients proteolyzed factor VIII (FIG. 2, shown for patients 1, 8 and 16) (Wootia et al. (2008) J Immunol 180:771 4-20). IgG did not proteolyze activated factor VII, prothrombin or serum albumin. In contrast, it was observed that IgG from some patients proteolyzed factor IX, yielding a major protein band migrating at 45 kDa (FIG. 3). IVIg (Intravenous Immunoglobulin) (FIG. 3) and IgG purified from the plasma of 7 patients with severe hemophilia B who have developed inhibitory anti-factor IX antibodies following therapeutic administration of factor IX did not proteolyze factor IX (Christophe et al. (2001) Stood 98:1416-23). The migration profiles of factor IX incubated with patients' IgG or with IVIg, were subjected to densitometric analysis so as to compute the specific rates of proteolysis (FIG. 4 and FIG. 5, Y-axes, Table 2). IVIg exhibited a marginal catalytic activity of 0.06±0.06 mmol/min/mol towards factor IX. Purified IgG from 36 of the 65 patients exhibited factor IX-proteolyzing activity that was significantly higher than that of IVIg (Table 2, P<0.05).
Table 2. Factor VIII inhibitory titers, mortality and specific rates of hydrolysis and activation of coagulation factors by IgG purified from the plasma of patients with acquired hemophilia.

<table>
<thead>
<tr>
<th>PATIENT</th>
<th>INHIBITORY TITER (BU/ml)a</th>
<th>MORTALITY OF PATIENTS&quot; (day)</th>
<th>HYDROLYSIS OF FACTOR VIII0 (mmol per min per mol IgG)</th>
<th>HYDROLYSIS OF FACTOR IXd (mmol per min per mol IgG)</th>
<th>ACTIVATION OF FACTOR IXs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>D (148)</td>
<td>0.45 ± 0.2*</td>
<td>0.03 ± 0.01</td>
<td>0.29 ± 0.05*</td>
</tr>
<tr>
<td>2</td>
<td>63</td>
<td>A (365)</td>
<td>0.15 ± 0.0</td>
<td>0.29 ± 0.45</td>
<td>0.96 ± 0.10*</td>
</tr>
<tr>
<td>3</td>
<td>128</td>
<td>D (54)</td>
<td>0.14 ± 0.1</td>
<td>0.44 ± 0.38*</td>
<td>0.07 ± 0.00*</td>
</tr>
<tr>
<td>4</td>
<td>114</td>
<td>D (9)</td>
<td>0.13 ± 0.1</td>
<td>0.37 ± 0.06*</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>5</td>
<td>380</td>
<td>nd</td>
<td>0.17 ± 0.0</td>
<td>0.35 ± 0.19*</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>6</td>
<td>32</td>
<td>A (365)</td>
<td>0.12 ± 0.1</td>
<td>0.37 ± 0.18*</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>7</td>
<td>49.4</td>
<td>nd</td>
<td>0.08 ± 0.0</td>
<td>0.49 ± 0.29*</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>8</td>
<td>3.1</td>
<td>D (196)</td>
<td>0.38 ± 0.2*</td>
<td>0.42 ± 0.14*</td>
<td>0.95 ± 0.32*</td>
</tr>
<tr>
<td>9</td>
<td>42</td>
<td>A (365)</td>
<td>0.13 ± 0.0</td>
<td>0.33 ± 0.26*</td>
<td>1.33 ± 0.34*</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>A (365)</td>
<td>0.17 ± 0.0</td>
<td>0.64 ± 0.42*</td>
<td>2.01 ± 0.56*</td>
</tr>
<tr>
<td>11</td>
<td>14</td>
<td>D (116)</td>
<td>0.08 ± 0.0*</td>
<td>0.01 ± 0.01</td>
<td>0.33 ± 0.12*</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>A (365)</td>
<td>0.12 ± 0.1</td>
<td>0.17 ± 0.13</td>
<td>0.70 ± 0.15*</td>
</tr>
<tr>
<td>13</td>
<td>40</td>
<td>A (365)</td>
<td>0.16 ± 0.1*</td>
<td>0.21 ± 0.02</td>
<td>0.21 ± 0.05*</td>
</tr>
<tr>
<td>14</td>
<td>2</td>
<td>A (365)</td>
<td>0.22 ± 0.0*</td>
<td>0.73 ± 0.02*</td>
<td>3.43 ± 0.48*</td>
</tr>
<tr>
<td>15</td>
<td>80</td>
<td>A (365)</td>
<td>0.18 ± 0.0*</td>
<td>0.32 ± 0.02</td>
<td>0.28 ± 0.06*</td>
</tr>
<tr>
<td>16</td>
<td>52</td>
<td>A (365)</td>
<td>0.14 ± 0.0*</td>
<td>1.04 ± 0.14*</td>
<td>3.49 ± 0.59*</td>
</tr>
<tr>
<td>17</td>
<td>100</td>
<td>A (365)</td>
<td>0.36 ± 0.1*</td>
<td>0.17 ± 0.08</td>
<td>0.42 ± 0.04*</td>
</tr>
<tr>
<td>18</td>
<td>10</td>
<td>A (365)</td>
<td>0.09 ± 0.0</td>
<td>0.36 ± 0.27*</td>
<td>0.13 ± 0.03*</td>
</tr>
<tr>
<td>19</td>
<td>1.4</td>
<td>D (25)</td>
<td>0.10 ± 0.0</td>
<td>0.38 ± 0.29*</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>20</td>
<td>18</td>
<td>D (4)</td>
<td>0.08 ± 0.0</td>
<td>0.54 ± 0.61*</td>
<td>0.19 ± 0.06*</td>
</tr>
<tr>
<td>21</td>
<td>1050</td>
<td>nd</td>
<td>0.15 ± 0.1*</td>
<td>0.35 ± 0.16*</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>22</td>
<td>330</td>
<td>D (211)</td>
<td>0.14 ± 0.1*</td>
<td>0.28 ± 0.05*</td>
<td>0.59 ± 0.07*</td>
</tr>
<tr>
<td>23</td>
<td>18</td>
<td>A (365)</td>
<td>0.30 ± 0.1*</td>
<td>0.97 ± 0.13*</td>
<td>1.78 ± 0.17*</td>
</tr>
<tr>
<td>24</td>
<td>1.3</td>
<td>D (71)</td>
<td>0.05 ± 0.0</td>
<td>0.50 ± 0.17*</td>
<td>0.59 ± 0.00*</td>
</tr>
<tr>
<td>25</td>
<td>60</td>
<td>D (54)</td>
<td>0.06 ± 0.0</td>
<td>0.16 ± 0.05</td>
<td>0.04 ± 0.00*</td>
</tr>
<tr>
<td>26</td>
<td>1</td>
<td>A (365)</td>
<td>0.06 ± 0.0</td>
<td>0.18 ± 0.07</td>
<td>0.20 ± 0.04*</td>
</tr>
<tr>
<td>27</td>
<td>4</td>
<td>A (365)</td>
<td>0.10 ± 0.1</td>
<td>0.27 ± 0.09*</td>
<td>0.42 ± 0.04*</td>
</tr>
<tr>
<td>28</td>
<td>56</td>
<td>A (21)</td>
<td>0.07 ± 0.0</td>
<td>0.08 ± 0.05</td>
<td>1.98 ± 0.17*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>D (365)</td>
<td>0.13 ± 0.0*</td>
<td>0.17 ± 0.07</td>
<td>0.14 ± 0.04*</td>
</tr>
<tr>
<td>30</td>
<td>1.5</td>
<td>A (365)</td>
<td>0.08 ± 0.0</td>
<td>1.10 ± 0.04*</td>
<td>0.89 ± 0.28*</td>
</tr>
<tr>
<td>31</td>
<td>3</td>
<td>A (365)</td>
<td>0.11 ± 0.1*</td>
<td>0.34 ± 0.08*</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>32</td>
<td>7</td>
<td>A (365)</td>
<td>0.19 ± 0.0*</td>
<td>0.39 ± 0.23*</td>
<td>2.13 ± 0.71*</td>
</tr>
<tr>
<td>33</td>
<td>362,7</td>
<td>nd</td>
<td>0.08 ± 0.0</td>
<td>0.10 ± 0.10</td>
<td>0.04 ± 0.01*</td>
</tr>
<tr>
<td>34</td>
<td>249,2</td>
<td>nd</td>
<td>0.09 ± 0.1</td>
<td>0.19 ± 0.18*</td>
<td>0.06 ± 0.03</td>
</tr>
<tr>
<td>35</td>
<td>62</td>
<td>A (365)</td>
<td>0.32 ± 0.0*</td>
<td>0.41 ± 0.22*</td>
<td>0.31 ± 0.03*</td>
</tr>
<tr>
<td>36</td>
<td>54</td>
<td>A (365)</td>
<td>0.21 ± 0.1*</td>
<td>0.13 ± 0.11</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>37</td>
<td>1</td>
<td>nd</td>
<td>0.13 ± 0.1*</td>
<td>0.11 ± 0.07</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>38</td>
<td>105,4</td>
<td>nd</td>
<td>0.10 ± 0.0</td>
<td>0.05 ± 0.04</td>
<td>0.04 ± 0.01*</td>
</tr>
<tr>
<td>39</td>
<td>92,5</td>
<td>nd</td>
<td>0.09 ± 0.0</td>
<td>0.14 ± 0.11</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>40</td>
<td>26,6</td>
<td>nd</td>
<td>0.10 ± 0.1</td>
<td>0.09 ± 0.08</td>
<td>0.03 ± 0.00*</td>
</tr>
<tr>
<td>41</td>
<td>10</td>
<td>D (230)</td>
<td>0.17 ± 0.0*</td>
<td>0.33 ± 0.02*</td>
<td>0.85 ± 0.13*</td>
</tr>
<tr>
<td>42</td>
<td>29,9</td>
<td>nd</td>
<td>0.10 ± 0.0</td>
<td>0.15 ± 0.12</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>43</td>
<td>4,9</td>
<td>A (365)</td>
<td>0.30 ± 0.2*</td>
<td>0.24 ± 0.16*</td>
<td>0.12 ± 0.03*</td>
</tr>
<tr>
<td>44</td>
<td>13</td>
<td>A (365)</td>
<td>0.10 ± 0.0</td>
<td>0.34 ± 0.17*</td>
<td>0.26 ± 0.09*</td>
</tr>
<tr>
<td>45</td>
<td>58,2</td>
<td>nd</td>
<td>0.17 ± 0.0*</td>
<td>0.25 ± 0.14*</td>
<td>0.03 ± 0.01*</td>
</tr>
<tr>
<td>46</td>
<td>25</td>
<td>A (365)</td>
<td>-</td>
<td>0.08 ± 0.03</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>47</td>
<td>35</td>
<td>A (365)</td>
<td>-</td>
<td>0.62 ± 0.08*</td>
<td>0.38 ± 0.03*</td>
</tr>
<tr>
<td>48</td>
<td>190</td>
<td>D (22)</td>
<td>-</td>
<td>0.07 ± 0.01</td>
<td>0.07 ± 0.00*</td>
</tr>
<tr>
<td>49</td>
<td>6,2</td>
<td>A (365)</td>
<td>-</td>
<td>0.02 ± 0.01</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>A (365)</td>
<td>-</td>
<td>0.32 ± 0.12*</td>
<td>0.22 ± 0.02*</td>
</tr>
<tr>
<td>51</td>
<td>5,5</td>
<td>A (365)</td>
<td>-</td>
<td>0.14 ± 0.06</td>
<td>0.09 ± 0.02*</td>
</tr>
<tr>
<td>52</td>
<td>13,6</td>
<td>A (365)</td>
<td>-</td>
<td>0.07 ± 0.02</td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>53</td>
<td>8</td>
<td>A (365)</td>
<td>-</td>
<td>0.17 ± 0.03*</td>
<td>0.16 ± 0.06*</td>
</tr>
<tr>
<td>54</td>
<td>3</td>
<td>A (365)</td>
<td>-</td>
<td>0.10 ± 0.02</td>
<td>0.12 ± 0.03*</td>
</tr>
<tr>
<td>55</td>
<td>1,5</td>
<td>A (32)</td>
<td>-</td>
<td>0.02 ± 0.01</td>
<td>0.06 ± 0.02*</td>
</tr>
<tr>
<td>56</td>
<td>28</td>
<td>A (365)</td>
<td>-</td>
<td>0.04 ± 0.03</td>
<td>0.08 ± 0.02*</td>
</tr>
<tr>
<td>57</td>
<td>27</td>
<td>A (365)</td>
<td>-</td>
<td>0.19 ± 0.07*</td>
<td>0.04 ± 0.00*</td>
</tr>
<tr>
<td>58</td>
<td>1,7</td>
<td>D (99)</td>
<td>-</td>
<td>0.12 ± 0.05</td>
<td>0.11 ± 0.04</td>
</tr>
<tr>
<td>59</td>
<td>4,4</td>
<td>A (365)</td>
<td>-</td>
<td>0.10 ± 0.02</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>60</td>
<td>98</td>
<td>A (365)</td>
<td>-</td>
<td>0.04 ± 0.01</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>61</td>
<td>45</td>
<td>A (365)</td>
<td>-</td>
<td>0.29 ± 0.06*</td>
<td>0.29 ± 0.05*</td>
</tr>
<tr>
<td>62</td>
<td>2</td>
<td>A (365)</td>
<td>-</td>
<td>0.28 ± 0.05*</td>
<td>0.20 ± 0.04*</td>
</tr>
<tr>
<td>63</td>
<td>1</td>
<td>A (365)</td>
<td>-</td>
<td>0.25 ± 0.02*</td>
<td>0.26 ± 0.07*</td>
</tr>
<tr>
<td>64</td>
<td>11,5</td>
<td>A (365)</td>
<td>-</td>
<td>0.39 ± 0.01*</td>
<td>0.38 ± 0.09*</td>
</tr>
</tbody>
</table>
Inhibitory titers were measured in plasma using the Bethesda assay.

Mortality was documented in the case of 54 of the 65 patients over a period of 365 days. Numerical within brackets indicate the number of days either between diagnosis and death, or until patients were documented alive for the last time. A, alive; D, deceased; nd, not documented.

The results are mean ± standard deviation of four independent experiments. Rates of factor VIII hydrolysis were calculated by densitometric analysis of Western blots. Spontaneous hydrolysis that occurred upon incubation of factor VIII in the presence of buffer alone was subtracted from each analysis. The mean coefficient of variation was 0.53±0.22 (range, 0.09 to 1.15).

The results are mean ± standard deviation of three to four independent experiments. Rates of factor IX hydrolysis were calculated by densitometric analysis of Western blots. Spontaneous hydrolysis that occurred upon incubation of factor IX in the presence of buffer alone was subtracted from each analysis. The mean coefficient of variation was 0.47±0.32 (range, 0.01 and 1.58).

The results are mean ± standard deviation of three independent experiments. Rates of factor IX activation were calculated in a functional assay as described (see Methods). The mean coefficient of variation was 0.26±0.15 (range, 0.00 and 0.55).

P<0.05 for the comparison with intravenous immunoglobulins (IVlg), using the analysis of variance post-hoc test.

No factor IX-binding IgG was detected in a factor IX-specific ELISA when whole plasma was used. In contrast, IgG purified from the plasma of 21 of 65 patients demonstrated binding activity with factor IX, which was greater than that of the mean±1 SD measured for IVlg. The scored anti-factor IX IgG titers correlated with the rates of IgG-mediated factor IX proteolysis (FIG. 4, P=0.384). Rates of IgG-mediated factor VIII proteolysis did not correlate with rates of IgG-mediated factor IX proteolysis (FIG. 5, P=0.688): purified IgG proteolyzed either factor VIII or factor IX, both molecules or none.

The proteolysis rate of ¹²⁵I-labeled factor IX by IgG from patients 16, 23 and 32 was then measured, in the presence of increasing concentrations of unlabeled factor IX. The curves of the reciprocal of the velocity plotted as a function of the reciprocal of the substrate concentration were linear (shown for patient 16, FIG. 6, inset, R=0.88), indicating that the reaction conformed to Michaelis-Menten kinetics. The calculated average Km and apparent Vmax for the reactions ranged between 0.94±0.25 and 7.26±3.99 µM and 0.30±0.08 and 0.82±0.1 9 nM/min, respectively (Table 3).
Table 3. Kinetic parameters of the hydrolysis of FIX by IgG of patients with acquired hemophilia

<table>
<thead>
<tr>
<th>Patients</th>
<th>Vmax (^a) (nM/min)</th>
<th>Km (^a) (µM)</th>
<th>Kcat (^b) (min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>0.82 ± 0.19</td>
<td>7.26 ± 3.99</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td>23</td>
<td>0.41 ± 0.03</td>
<td>0.94 ± 0.25</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>32</td>
<td>0.30 ± 0.03</td>
<td>1.81 ± 0.61</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>Mean</td>
<td>0.51 ± 0.08</td>
<td>3.34 ± 1.62</td>
<td>0.11 ± 0.02</td>
</tr>
</tbody>
</table>

\(^a\)Factor IX was incubated at increasing concentrations (0 to 20 µM) with IgG (167 nM) of three patients with acquired hemophilia for 24 hr at 37°C. Hydrolysis rates were computed and the data were fitted to the Michaelis-Menten equation to derive the apparent Vmax and average Km. The data represent the mean ± standard error of three independent experiments.

\(^b\)The nominal Kcat value is the sum of the individual constants of different antibodies within the polyclonal preparation. In the calculation of Kcat, the concentration of catalytic sites was estimated to be 4.67 nM (i.e., 140 µg of antigen-specific IgG for 10 mg IgG).
EXAMPLE 3: Determination of the activation of factor IX by IgG

METHODS

Activation of factor IX

The IgG-mediated activation of factor IX was measured by its ability to activate factor X. Factor IX (800 nM) was incubated with isolated IgG (54 nM) for 23 hr at 37°C. The mixture was then incubated with factor X (1 µM) (Kordia, Leiden, the Netherlands), a saturating concentration (30 µM) of phospholipids vesicles and CaCl₂ (5 mM) for 1 hr at 37°C. Phospholipid vesicles (PC:PS, 3:1) of nominal 100 nm-diameter were synthesized by the membrane extrusion method (Olson et al. (1979) Biochim Biophys Acta 557:9-23). Phospholipid concentration was determined by phosphate analysis. Factor X activation was stopped by addition of 0.1 M EDTA. Activated factor X formation was determined by measuring the amyloolytic activity towards the synthetic substrate S2366 (0.5 mM, DiaPharma Group, Inc., Ohio, USA). During the assay, less than 5 percent of factor X was converted to activated factor X, and activated factor X formation was linear. Substrate conversion was monitored at 405 nm. Concentrations of generated activated factor X were determined from a standard curve derived from the cleavage rate of factor X by known concentrations of activated factor IX under the same conditions.

Analysis of N-terminal sequences.

Unlabelled recombinant factor IX (4 µg) was treated for 24 hr at 37°C with patients’ IgG (1 µg) in 40 µl of catalytic buffer. The resultant factor IX fragments were separated by 10 percent SDS-PAGE at 50 mA in non-reducing conditions and were transferred for 2 hr at 50 mA onto a Hybond-P PVDF membrane (GE Healthcare) in 10 mM CAPS, 10 percent ethanol at pH 11.0. After being stained with amido black, visible bands were cut and subjected to N-terminal sequencing, using an automatic Procise 610A Protein Sequencer (Applied Biosystems, Foster City, CA, USA). The amount of protein sequenced ranged from 4 to 34.8 pmoles.

RESULTS

Using unlabelled factor IX, an electrophoretic separation was performed in non-reduced conditions (FIG. 7) of factor IX (lane 1). IgG from patients 16 and 32
(lanes 2 and 3), activated factor IX (lane 4) and factor IX pre-incubated in the presence of IgG from patients 16 and 32 (lanes 5 and 6) for 24 hours. Incubation of unlabelled factor IX with patients' IgG generated a protein band with a molecular weight identical to that of activated factor IX (45 kDa). N-terminal amino-acid sequencing of the 45 kDa protein band generated upon proteolysis of factor IX by the IgG from patients 10, 14, 16, 23 and 32 revealed the presence of 2 protein sequences: YNSG and VVGG. YNSG amino-acid sequence represents the N-terminal end of factor IX. VVGG sequence corresponds to the N-terminal part of the catalytic domain of factor IX, a cleavage site for factor IX-activating enzymes.

To investigate whether IgG mediate activation of factor IX, a functional assay was developed wherein the factor IX proteolyzed by patients' IgG was incubated in the presence of factor X. The activation of factor X by the generated activated factor IX was monitored using a specific substrate. Importantly, the assay was independent of the presence of factor VIII, the co-factor of activated factor IX, so as to prevent interference by factor VIII-inhibitory IgG. Purified IgG neither directly activated factor X nor hydrolyzed the chromogenic substrate. Incubation of factor IX alone, in the presence of IVIg or in the presence of IgG from an inhibitor-positive patients with hemophilia B (HJC), yielded marginal levels of activated factor IX (FIG. 8, less than 0.02 mmol/min/mol). In contrast, IgG from 49 of the 65 acquired hemophilia patients demonstrated a statistically significant activation of factor IX as compared to IVIg (Table 2, P<0.05), with values ranging from 0.02 to 3.49 mmol/min/mol. The specific rates of IgG-mediated activation of factor IX demonstrated a positive and significant correlation with the rates of IgG-mediated proteolysis of factor IX (FIG. 9, P<0.001).

It is here demonstrated that the IgG from some patients with acquired hemophilia significantly proteolyze and activate factor IX.

**EXAMPLE 4: Relevance of IgG-mediated activation of factor IX**

**METHOD**

**Thrombin Generation Assay**

Human plasma deficient in factor VIII and devoid of platelets (PPP) (Dade-Behring, Siemens Diagnostics, Eschborn, Germany) was used. Factor VIII was supplemented to factor VIII-deficient plasma at 0, 3, 10 or 30 percent (0, 9, 30 and
90 pM) of the values in normal plasma. Activated factor IX was used at 0 and 0.3 nM diluted in factor VIII reconstituted plasma. Briefly, 80 µl of each test sample were plated, followed by addition of 20 µl of HEPES buffer or thrombin calibrator (Synapse BV, Maastricht, the Netherlands) on micro-titer plates. The plates were then transferred to a fluorimeter (Thermo Scientific) pre-heated to 37°C. The generation of thrombin was triggered by adding 20 µl of substrate containing 10 mM CaCl$_2$. The kinetic of thrombin generation was monitored for 120 min using a calibrated automated thrombogram (Thrombinscope BV, Maastricht, the Netherlands).

RESULTS

In this cohort, the cumulative mortality one year following diagnosis of acquired hemophilia was 24.6 percent, 95% CI [15.1 - 38.7]. The predictive value of IgG-mediated factor IX activation was explored by bivariate analysis of survival using Kaplan-Meyer (FIG. 11). Survival was significantly lower in men (P=0.019), and tended to be lower in elderly individuals (P=0.112), in patients with cancer (P=0.079), in patients with residual FVIII below 1 percent (P=0.221), and in patients with the lowest rates of IgG-mediated activation of factor IX (P=0.293). No association was observed between survival and the inhibitory titer (P=0.408) or aPTT (P=0.632). The trend towards an association between IgG-mediated factor IX activation at the time of diagnosis and survival at one year was confirmed in multivariate analysis (P=0.161, Table 4).

Table 4. Multivariate analysis (Cox model) of the survival of patients with acquired hemophilia one year following diagnosis

<table>
<thead>
<tr>
<th></th>
<th>Nb$^a$</th>
<th>Adjusted RR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>35</td>
<td>1</td>
<td></td>
<td>0.106</td>
</tr>
<tr>
<td>Female</td>
<td>19</td>
<td>0.18</td>
<td>[0.02-1.44]</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td>0.185</td>
</tr>
<tr>
<td>&lt;65</td>
<td>18</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>65 - 79</td>
<td>21</td>
<td>1.40</td>
<td>[0.22-8.81]</td>
<td></td>
</tr>
<tr>
<td>≥80</td>
<td>15</td>
<td>3.82</td>
<td>[0.79-18.51]</td>
<td></td>
</tr>
</tbody>
</table>
The potency of activated factor IX to restore thrombin generation was then investigated in the context of reduced factor VIII levels. Patients' IgG were estimated to generate 0.3 nM of activated factor IX in 24 hours, based on the calculated average kinetic parameters (Table 3). Besides, due to the presence of factor VIII-inhibiting antibodies, the patients included in this study had residual levels of circulating factor VIII comprised between <1 and 30 percent of the value found in normal plasma (Table 1). In the absence of activated factor IX, no thrombin generation was detected even in the presence of 30% residual factor VIII. Conversely, addition of 0.3 nM activated factor IX to plasma restored thrombin generation provided the presence of ≥ 3 percent residual factor VIII (FIG. 10): the time-to-peak was 42.5, and 27.8 and 22.6 min when factor VIII was supplemented at 3, 10 and 30 percent to FVIII-deficient plasma, respectively; it was infinite in the absence of FVIII.

Of note, a trend towards a correlation between FVIII activity in plasma at the time of diagnosis and survival after one year was observed (FIG. 11 and Table 4).

Thus, it is here observed that IgG from 75 percent of the patients significantly activate factor IX as compared to IVIg. Patients with elevated rates of factor IX activation at the time of diagnosis demonstrated a tendency towards increased survival one year later.
In this cohort, the cumulative mortality was 24.6 percent. A complete follow-up of the patients over 365 days was available for 54 patients. The predictive value of our in vitro assay was suggested by the trend towards an association between IgG-mediated factor IX activation at the time of diagnosis, and survival of the patients during the following year. The power of our analyse is reduced owing to the relatively limited number of available patients with acquired hemophilia and various potential causes of death inherent to several etiologies of this disease (Table 1). Taken together, the data suggest that, in certain underlying pathologies, the presence of factor IX-activating IgG is beneficial; IgG-mediated factor IX activation may represent an anti-hemorrhagic mechanism that compensates, at least in part, for the inhibition of endogenous factor VIII by the patients' anti-factor VIII autoantibodies. In support, in vitro addition of picomolar levels of activated factor IX to plasma was able to partly restore thrombin generation, provided that residual factor VIII in plasma was ≥3 percent.

Hemorrhages in patients with acquired hemophilia are treated with factor VIII, activated factor VII or activated pro-thrombin complexes, all of which are hampered by short half-life and/or risks for thromboembolic complications. These results raise the issue of the therapeutic relevance of the passive administration of factor IX-activating antibodies; catalytic antibodies would advantageously combine the capacity for "turn-over" that characterizes enzymatic activities, low risk for thrombotic complications owing to their low catalytic rates of factor IX activation, with long half-life typical of IgG molecules. Such a therapy would be of particular interest for patients with negative survival prognosis based on low circulating levels of factor IX-activating IgG and circulating factor VIII.
1. An antibody directed against coagulation factor IX, wherein said antibody catalyzes proteolysis of coagulation factor IX and yields activated coagulation factor IX.

2. The antibody of claim 1, wherein the antibody proteolyses coagulation factor IX between amino acids Arg^{145} and Ala^{146} and/or Arg^{180} and Val^{181} of SEQ ID NO: 1.

3. The antibody according to claim 1 or 2, wherein the antibody is selected from the group consisting of a polyclonal antibody, a monoclonal antibody, a chimeric antibody, a humanized antibody, and an antibody fragment including Fv, Fab, F(ab')_{2}, Fab', dsFv, scFv, sc(Fv)_{2} and diabodies.

4. The antibody according to any one of claims 1 to 3, wherein the antibody is an IgG.

5. A composition comprising an antibody according to any one of claims 1 to 4, together with a pharmaceutically acceptable carrier.

6. An antibody according to any one of claims 1 to 4, for use in the treatment of a blood coagulation disorder in an individual.

7. The antibody for use according to claim 6, wherein the blood coagulation disorder is selected from the group consisting of acquired hemophilia, hemophilia A, hemophilia B and hemophilia C.

8. The antibody for use according to claim 6 or 7, wherein the individual is at risk of excessive bleeding during surgery, delivery or menstruation.

9. A combination of at least two antibodies according to any one of claims 1 to 4, wherein at least one antibody is able to cleave the bond between Arg^{145} and Ala^{146}.
of SEQ ID NO: 1 and at least one antibody is able to cleave the bond between Arg^{180} and Val^{181} of SEQ ID NO: 1.

10. The use of an antibody as defined in any one of claims 1 to 4, for the in vitro prognosis of the outcome of acquired hemophilia.

11. A method of prognosing the outcome of acquired hemophilia in an individual comprising the steps consisting of:
   a) determining the level of antibodies directed against coagulation factor IX, wherein said antibodies catalyze proteolysis of coagulation factor IX and yield activated coagulation factor IX, or rate of conversion of coagulation factor IX into activated coagulation factor IX, in a biological sample of said individual;
   b) comparing said level or rate to at least one predetermined value; and
   c) predicting the outcome of the disease based upon the comparison of said level or rate with the predetermined value.

12. The method according to claim 11, wherein the predetermined value is a single value and a level of antibodies directed against coagulation factor IX or rate of conversion of coagulation factor IX into activated coagulation factor IX lower than the predetermined value is indicative of a low probability of survival within 1 year.

13. The method according to claim 11 or 12, wherein the predetermined value corresponds to a null level and the absence of said antibodies directed against coagulation factor IX value is indicative of a low probability of survival within 1 year.

14. The method according to any of claims 11 to 13, wherein said antibodies directed against coagulation factor IX are IgGs.

15. The method according to any of claims 11 to 14, which further comprises monitoring the level of antibodies directed against coagulation factor IX or the rate of conversion of coagulation factor IX into activated coagulation factor IX in the course of time, wherein an increase of the level of said antibodies directed against
coagulation factor IX or of the rate of conversion of coagulation factor IX into activated coagulation factor IX in the course of time is indicative of a higher probability of survival within 1 year.
FIG. 2

FIG. 3
**FIG. 6**

<table>
<thead>
<tr>
<th>Lanes</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>kDa</td>
<td>250</td>
<td>150</td>
<td>100</td>
<td>75</td>
<td>50</td>
<td>37</td>
</tr>
</tbody>
</table>

**FIG. 7**

<table>
<thead>
<tr>
<th>Patient</th>
<th>FIX</th>
<th>Patients' IgG</th>
<th>FIXa</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>32</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>32</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K16/36 A61P7/04 C07K16/40 A61K39/395

ADD.

According to International Patent Classification (IPC) or both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>LACROIX-DESMAZES SEBASTIEN ET AL: &quot;High levels of catalytic anti bodies correlate with favorable outcome in sepsis&quot;., PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 102, no. 11, March 2005 (2005-03), pages 4109-4113, XP002582358, ISSN: 0027-8424 figure 4</td>
<td>1-5, 9</td>
</tr>
</tbody>
</table>

X Further documents are listed in the continuation of Box C.

X See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another document, or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"*" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

Date of the actual completion of the international search
11 March 2011

Date of mailing of the international search report
21/03/2011

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax. (+31-70) 340-3016

Authorized officer
Vadot, Pierre
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>WOOTLA BHARATH ET AL: &quot;Hydrolysis of coagulant factors by circulating IgG is associated with a reduced risk for chronic allograft nephropathy in renal transplant patients&quot;, JOURNAL OF IMMUNOLOGY, vol. 180, no. 12, June 2008 (2008-06), pages 8455-8460, XP002582359, ISSN: 0022-1767, the whole document</td>
<td>1-5, 9</td>
</tr>
<tr>
<td>A</td>
<td>&quot;WOOTLA BHARATH ET AL: &quot;Hydrolysis of coagulant factors by circulating IgG is associated with a reduced risk for chronic allograft nephropathy in renal transplant patients&quot;, JOURNAL OF IMMUNOLOGY, vol. 180, no. 12, June 2008 (2008-06), pages 8455-8460, XP002582359, ISSN: 0022-1767, the whole document</td>
<td>1-15</td>
</tr>
</tbody>
</table>

C(C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Form PCT/ISA/210 (continuation of second sheet) (April 2008)
<table>
<thead>
<tr>
<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
<th>Publication date</th>
</tr>
</thead>
<tbody>
<tr>
<td>WO 0119992 A2</td>
<td>22-03-2001</td>
<td>AT 411997 B</td>
<td>26-08-2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AT 365799 T</td>
<td>15-07-2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AU 780775 B2</td>
<td>14-04-2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AU 7775900 A</td>
<td>17-04-2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2384660 A1</td>
<td>22-03-2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 1390258 A</td>
<td>08-01-2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CZ 20020935 A3</td>
<td>14-08-2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DE 60035356 T2</td>
<td>14-02-2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 1220923 A2</td>
<td>10-07-2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ES 2288873 T3</td>
<td>01-02-2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HU 0203750 A2</td>
<td>28-02-2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 4313531 B2</td>
<td>12-08-2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 2003509049 T</td>
<td>11-03-2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 2006111638 A</td>
<td>27-04-2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PL 354976 A1</td>
<td>22-03-2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SK 3662002 A3</td>
<td>02-07-2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 7033590 B1</td>
<td>25-04-2006</td>
</tr>
</tbody>
</table>