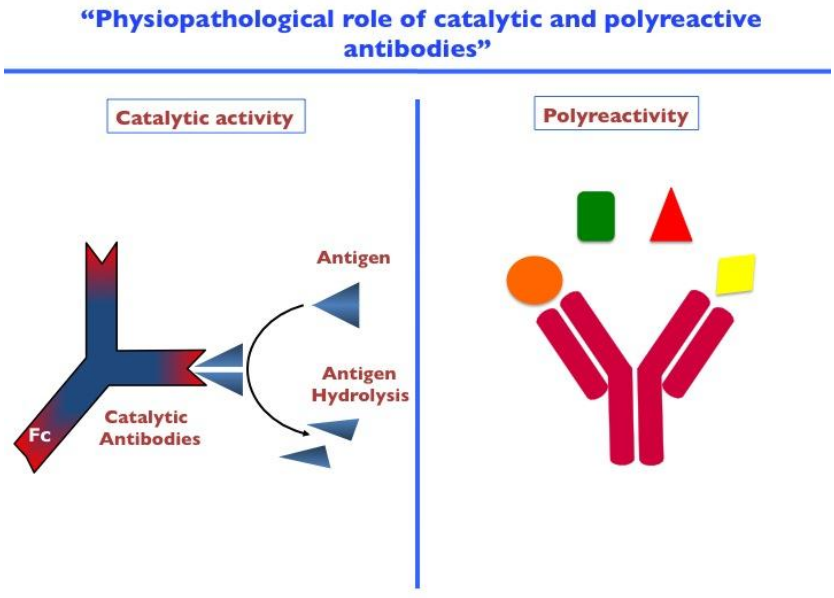
Par **Ankit MAHENDRA**

Rôle physiopathologique des anticorps catalytiques et des anticorps polyréactifs

Thèse présentée pour l'obtention du grade de Docteur de l'UTC



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Sujet de la thèse

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Thèse dirigée par : Dr. Sébastien LACROIX-DESMAZES

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Soutenue le : le 29 Janvier 2013

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Résumé en Français

Rôle physiopathologique des anticorps catalytiques et des anticorps polyréactifs

par

Ankit MAHENDRA

<< Biotechnologie et mise en œuvre des Fonctions Biologiques >>

Thesis is presented at the Faculty of the Université de Technologie de Compiègne for obtaining the degree of Doctor of Philosophy (Ph.D) of the Université de Technologie de Compiègne

Les mot-clés en Français – anticorps catalytiques, abzymes, anticorps polyréactifs, hémophlie acquise, transplantation rénale, facteur VIII, facteur IX

Les anticorps sont les molécules effectrices de l'immunité adaptatrice humorale. Ils se lient spécifiquement et neutralisent une large panoplie d'antigènes. Au-delà de leurs fonctions classiques, les anticorps possèdent les propriétés moins explorées que sont l'activité catalytique, qui permet aux anticorps de se comporter comme des enzymes, et la polyréactivité, qui représente la capacité d'une molécule d'anticorps à se lier à plusieurs antigènes structurellement différents.

Les anticorps catalytiques sont retrouvés dans plusieurs pathologies chez l'homme, telle que l'hémophilie acquise, une maladie caractérisée par la survenue d'autoanticorps anti-facteur VIII. Dans ce travail, nous décrivons des IgG hydrolysant et activant le facteur IX de la coagulation chez les patients avec hémophilie acquise. Par ailleurs, nous avons effectué une étude longitudinale de deux ans des IgG catalytiques chez les patients subissant une transplantation rénale.

Les anticorps polyréactifs représentent une proportion importante du répertoire des immunoglobulines circulantes. De plus, les sites inflammatoires sont abondants en molécules, telles que l'hème libre, capables de rendre polyréactives certaines IgG monoréactives. Nous avons étudié l'influence de la nature des régions constantes de la chaîne lourde des anticorps sur leur susceptibilité à devenir polyréactifs.

Ce travail apporte un nouvel éclairage sur l'importance physiopathologique des anticorps catalytiques et polyréactifs.

Résumé en Anglais

Physiopathological role of catalytic and polyreactive antibodies

by

Ankit MAHENDRA

<< Biotechnologie et mise en œuvre des Fonctions Biologiques >>

Thesis is presented at the Faculty of the Université de Technologie de Compiègne for obtaining the degree of Doctor of Philosophy (Ph.D) of the Université de Technologie de Compiègne

Les mot clés en Anglais – Catalytic antibodies, abzymes, polyreactive antibodies, acquired hemophilia, renal transplant, factor VIII, and factor IX

Antibodies are effector molecules of the humoral arm of the adaptive immune system that bind specifically and neutralize diverse array of antigens. Beyond the classical function of antibodies exist the relatively less explored properties, of “catalytic activity” that enable antibodies to act as enzymes and “polyreactivity” that confers the ability to bind to several structurally unrelated antigens.

Catalytic antibodies have been associated with several autoimmune, inflammatory and infectious diseases. Acquired hemophilia is an autoimmune disease, reported with the presence of catalytic antibodies against coagulation factor FVIII. In the present work, we have investigated the presence of factor IX (FIX) hydrolyzing IgG in patients with acquired hemophilia. We investigated the molecular mechanism and the physiological relevance of FIX activation upon hydrolysis by patients’ IgG. In addition, a longitudinal follow-up for 2 years was done in patients undergone renal transplant to investigate the evolution of catalytic antibodies in the course of disease.

Polyreactive antibodies constitute a major portion of the natural antibody repertoire. Additionally, sites of inflammation are abundant in protein destabilizing agents like free heme that can induce polyreactivity in monoreactive antibodies. We have investigated the effect of the antibody constant domain on heme-induced polyreactivity.

The present work has allowed us a better understanding of the physiopathological relevance of catalytic and polyreactive antibodies.

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Abbreviations

Ig	Immunoglobulin
Fab	Fragment, antigen binding
Fc	Fragment, crystallisable
V region	Variable region
D region	Diversity region
J region	Junction region
VL	Variable light chain
VH	Variable heavy chain
CDR	Complimentary determining region
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
ADCC	Antibody-dependent cellular cytotoxicity
CDC	Complement dépendent cytotoxicity
pH	Potential of hydrogen
mAbs	Monoclonal antibodies
CRA	Covalently reactive analog
VIP	Vasoactive intestinal peptide
ELISA	Enzyme linked immunosorbent assay
CAN	Chronic allograft nephrophathy
AHA	Acquired hemophila A

SLE	Systemic lupus erythematosus
HT	Hashimoto’s throiditis
MS	Multiple sclerosis
scFv	Single chain fragment variable
TSA	Transition state analog
Tg	Thyroglobulin
PFR-MCA	Proline-phenylalanine-arginine-methylcoumarineamide
MBP	Myelin basic protein

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Introduction

Introduction

Immunoglobulins (Ig) are glycoproteins that act as effector molecules of the humoral immune system. They are the end-product of humoral arm of the adaptive immune response. Immunoglobulins are molecules widespread among leaving species and are believed to be present early in ontogeny, as shown by their presence in lower vertebrates like sharks and birds. They are commonly acknowledged for their implication in eliminating pathogens and neutralizing toxins. This process is permitted by the exquisite specificity for single molecular structures (epitopes on antigens) and the large repertoire of different specificities, which in theory covers the entire array of foreign molecules that the organism may potentially confront.

Beyond the classical view/understanding of immunoglobulins, lies an array of functions, which are still confidential to a minor portion of the scientific community. This includes in particular, their seminal role in innate immunity that relies on the capacity of some Ig to bind several structurally unrelated antigens (referred to as “polyspecific antibodies”) and their capacity to behave as enzymes (referred to as “catalytic antibodies”).

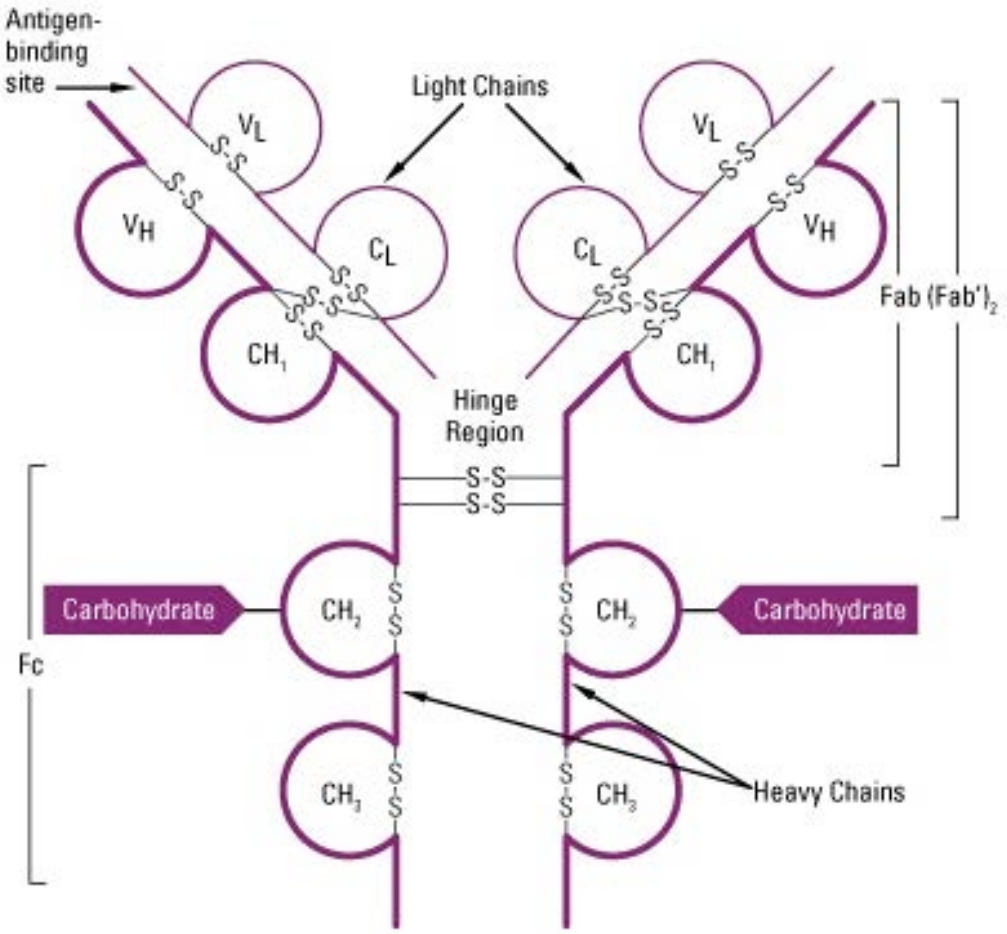
The main focus of my thesis work was concentrated on the latter properties of immunoglobulins.

1. Immunoglobulins

1.1. Structure of immunoglobulins

Immunoglobulins (Ig) are heterodimeric glycoproteins that consist of two heavy and two light polypeptide chains and belong to the Ig-domain superfamily (Figure 1). The molecular weight of the heavy chain is 50 kDa for immunoglogulins IgA, IgD and IgG, or 75 kDa for IgM and IgE. The molecular weight of the light chain is 25 kDa. The heavy and light chains interact with each other by strong covalent forces in the form of disulphide linkages as well as by non-covalent bonds such as hydrophobic interactions, salt bridges and hydrogen bonding. N-linked glycosylation is a common feature of Igs and is known to play an important role in Ig structure and function. However, O-linked glycosylation is less common and found only in the IgD and IgA1 class of antibodies (Mattu et al., 1998). Although glycosylation is the main feature of the constant region, it is often present in the variable region as well where it may affect secretion, half-life, antigen-binding and organ targeting. The predominant classes of immunoglobulins found in soluble form in the circulation are IgG>IgM>IgA>IgE. Surface-expressed immunoglobulins constitute the B-cell receptor (BcR). While immunoglobulins of the IgD, IgE and IgG class always circulate as monomers, circulating IgM are pentameric, and IgA primarly exist as monomer or dimer and, to a lesser extent, as trimer or tertramer. The multimeric form of Igs is structured by a linker polypeptide of 15 kDa, referred to as J-chain (Waldmann, 1991).

Figure 1. Structure of immunoglobulin



Structure of the heavy chain

The heavy chain encompasses a 110-amino acid-long, highly variable region and a constant region that is 330-440-amino acid-long depending on the antibody isotypes. The isotypes have been designated by Greek letters: γ, δ, α, μ, and ε, thus defining the IgG, IgD, IgA, IgM and IgE classes of the antibodies, respectively.

The variable region of the antibody heavy chain consists of two main parts; (1) hypervariable regions (HVs), which are also called complementarity-determining regions (CDRs) due to their complementarity towards antigen epitopes; (2) framework regions (FRs), that exhibit far less variations in their amino acid sequences. The constant region of the antibody heavy chain consists of several domains referred as CH_1, CH_2, CH_3 and CH_4. The antibody classes IgG, IgD, IgA contain three constant domains, whereas IgM and IgE consist of four constant domains. The C_H1 and C_H2 domains are separated by a hinge region that provides some flexibility to the molecule (Kuby et al, 2000).

Structure of the light chain

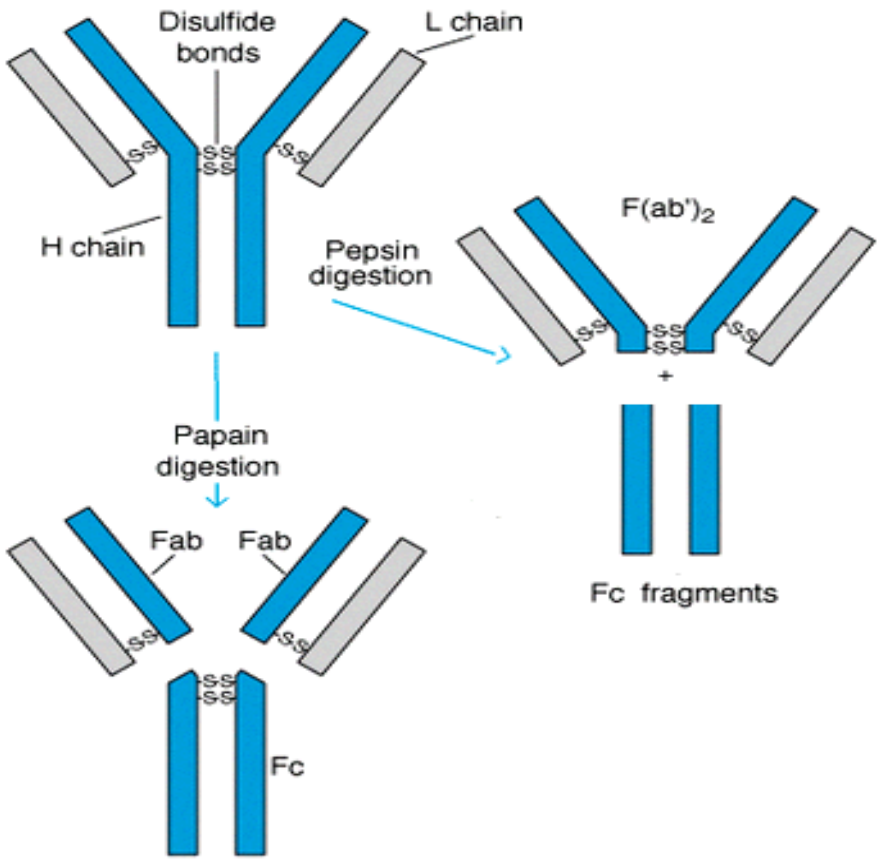
The length of the light chain is between 211-217 amino acids, of which the variable region is constituted of 110 amino acids. As the heavy chain, the light chain variable region also consists of similar number of CDRs and FRs. The light chain consists of only one constant domain. The constant domain is classified into two classes; kappa (k) and lambda (λ). The percentage distribution of kappa and lambda chains is 60% and 40% in humans, whereas, in mice it is 95% and 5% (Kuby et al, 2000). The light chain constant region is composed of only one constant domain referred as C_L.

From a functional perspective, Igs are bipolar molecules. The N-terminal end of the molecule (of both heavy and light chains) is implicated in antigen binding. The binding activity is resumed in the combined VH and VL regions. The binding part of antibodies can be isolated from the rest of the molecule by enzymatic digestion (Figure 2). Thus, digestion of IgG with pepsin generates a so-called $F(ab')_2$ fragments of 110 kD that contain two molecules each of VH-CH_1-hinge and VL-Ck/λ bound by disulfide bridges.

Conversely, IgG digestion by papain generates Fab fragments of 50 kD that contain one VH- CH_1 region attached to a VL- Ck/λ.

The C-terminal half of the molecule (the CH_2-$CH_{3/4}$ domains) mediates interactions with other molecules or cells of the immune system, including complement molecules and Fc-receptors. The C-terminal part of the molecule is referred to as the Fc portion of the immunoglobulin.

Figure 2. Enzymatic digestions of immunoglobulin molecule



1.2. Generation of a diverse repertoire of immunoglobulins

The vertebrate immune system has a remarkable ability to generate antibodies against a enormous diversity of antigens. This capability is ensured by a dynamic genetic machinery that generates more than 10^{10} combinations of immunoglobulin gene arrangements. However, the number of immunoglobulin gene segments is very limited (Nemazee, 2006). The vertebrate B cells employ several mechanisms that allow them to generate large array of antibodies from a relatively small number of immunoglobulin genes. These mechanisms include variable-region gene recombination, junctional diversity and random pairing.

Variable-region gene recombination:

The first level of generation of diversity is by random rearrangement of VDJ/VD genes. The variable region of the heavy chain is encoded by three genes, which are referred as variable (V), diversity (D) and joining (J) genes (Nemazee, 2006). The light chain is encoded by the V and J genes. The human gene repertoire encompasses 51 VH, 27 DH and 6 JH genes, as well as 40 VL and 5 JL genes. The rearrangement of variable region genes occurs during the ontogeny of B cells in the bone marrow by random recombinational events that lead to the shuffling of the immunoglobulin V(D)J genes. This is an orderly process in which first the heavy chain rearrangement takes place followed by that of the light chain. The rearrangement of the V(D)J gene segments generates the diversity installed in the variable regions, which are otherwise referred as complementarity determining regions (CDRs).

Junctional diversity

The second level of generation of diversity is by junctional diversity. It is a process followed by the V(D)J gene recombination event. The V(D)J gene rearrangement event leaves breaks due to cleavage of the unwanted gene segments. These breaks need to be filled to join the different variable region gene segments. Several proteins like the recombination activating gene-1 and 2 (RAG-1 and RAG-2), DNA repair protein Artemis, terminal deoxynucleotide transferase (TdT) and exonuclease work in concert to add/remove nucleotides for the proper joining of the variable region gene segments (Kim et al., 2000). The addition of nucleotide introduces a three-fold increase in the diversity of the variable region gene repertoire.

Random pairing of heavy and light chains

The third level of generation of diversity is ensured by random pairing of independently rearranged heavy and light chains.

Somatic hypermutation

Somatic hypermutation is an additional diversity generation mechanism that occurs in the course of an ongoing immune response, by which B cells alter their antigen specificity. It includes introduction or replacement of alternative nucleotides within the immunoglobulin VJ or VDJ segments. Somatic hypermutation of B cells occurs in germinal centres upon interaction with antigen-specific T cells. It is a random process, which may occur in the whole variable region, but is actually clustered in the CDRs. This unique mechanism allows selection of B cells with higher-affinity BcR for survival

because of their greater affinity towards antigen epitopes. The process is also referred as affinity maturation (Diaz and Casali, 2002).

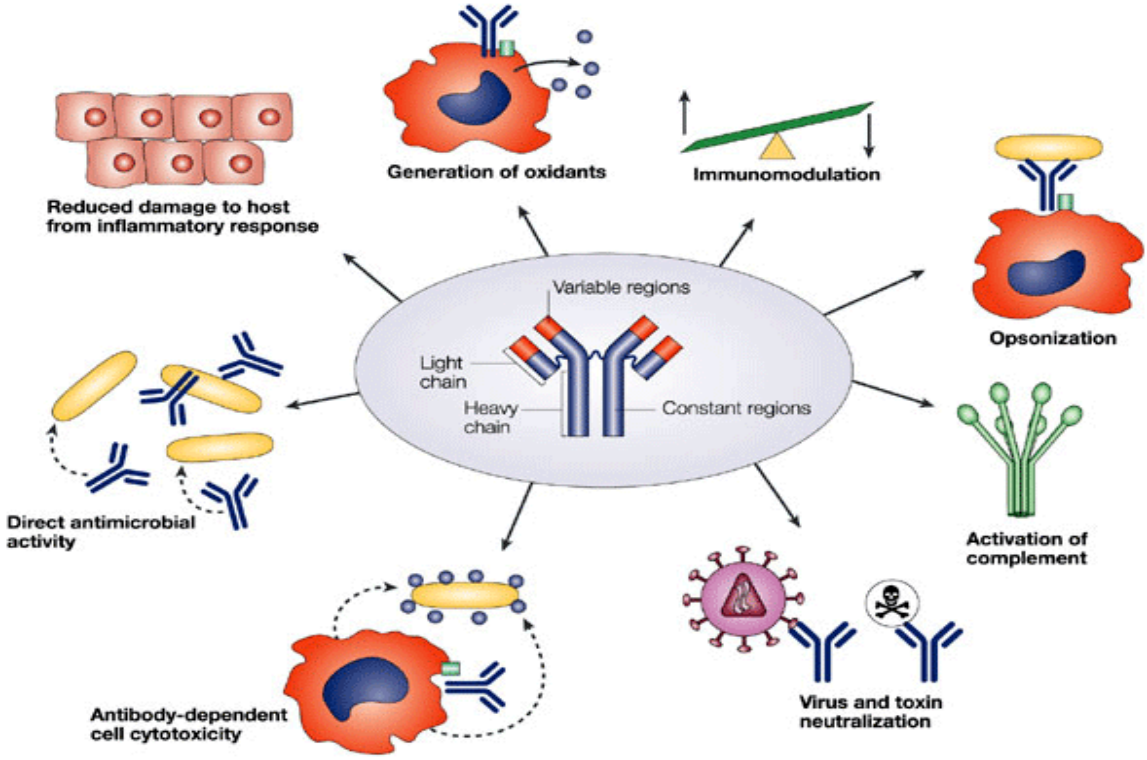
Class switching

Class switching or isotype switching is a process which occurs after the VDJ arrangement has completed. Under this event, the constant μ chain region (Cμ) downstream of the rearranged VDJ gene segment, is replaced by a new constant heavy chain (CH) gene segment (Market and Papavasiliou, 2003). This occurs through a deletional recombination mechanism in the switch region that is 5' to the CH region. Isotype switching is shown to be promoted by T cells, by secretion of cytokines and in a cell-to-cell contact-dependent manner. The cytokines mainly involved in class switching are IL-4 and TGF-β, whereas CD40 expressed on B cells influence the contact-dependent mechanism (Allen et al., 1993). The class switching procedure allows the B cells to produce antibodies of different isotypes, IgG, IgA and IgE, in which the antigen specificities of the antibodies still remain the same with changes only in the constant regions. The isotype determines the distribution of the antibody in the organism, by enabling it to diffuse through the epithelia and bind to specific cell-surface receptors, and hence dictates the very function of the immunoglobulin.

1.3. Functions of immunoglobulins

Antibodies are versatile molecules, which can perform a large array of functions. While immunoglobulin Fab regions bind to the antigen, the constant region interacts with cell surface receptors and other proteins for the elimination of pathogens or toxins (Figure 3).

Figure 3. Functions of immunoglobulins



Neutralization and agglutination: Antibodies can neutralize the activity of pathogens (microbes and viruses) (Crowe et al., 2001) or toxins (Chippaux) by directly binding to their target antigens. The ability of binding multiple antigens through the Fab region results in clumping of the antigens, referred to as agglutination.

Opsonization: Antibodies have the capacity of covering antigens or pathogens, a process called opsonization. The Ig in the formed complex interacts with phagocytic cells by means of their constant regions (Fc) with cell-surface receptors called the Fc receptors (FcRs). The binding of antigen-bound antibodies with multiple FcRs results in the cross-linking of the cell-surface receptors, which then transmits a signal to the cell resulting in the phagocytosis of the antigen/pathogen. The ingested antigen/pathogen is then degraded intracellularly.

Activation and fixation of complement: Antibodies, when bound to the surface of a pathogen, bind the components of the complement system. Binding of the first complement molecule C1q initiates the complement cascade, which finally results in the generation of the membrane-attack complex, resulting in the perforation of the membrane of the pathogen. Also, by-products of the complement cascade such as C3b can bind to antigen-antibody complexes and facilitate engulfment by macrophages upon interaction with CR1, the receptor for C3b on the cell surface (Paul, 2008).

Effector functions of the immunoglobulin G (IgG): Immunoglobulin G is the most abundant class of antibodies in the serum and accounts for nearly 75% of the total immunoglobulin in the serum. They are divided into 4 subclasses IgG1, IgG2, IgG3 and IgG4, based on the number of amino acids in their hinge regions. These changes in the hinge region affect the biological activity of the antibody subclasses. IgG1, IgG2 and IgG3 are efficient in transcytosis through the placenta. Among the 4 subclasses IgG3 is the most efficient activator of complement, whereas IgG4 does not bind with complement at all. Both IgG1 and IgG3 have a good affinity to Fc receptors, IgG4 has intermediate affinity, and IgG2 has the least affinity. IgG molecules mediate their effector functions by

interaction of their Fc region with FcγRs on the cell surfaces. The two mechanisms by which IgGs mediate their effector functions are antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Figure 3). The increasing order of effector function of IgG subclasses for ADCC and CDC is IgG4 < IgG2 < IgG1 ≤ IgG3 (Paul, 2008).

IgG-mediated catalytic activity: The antigen-antibody interaction is in many ways similar to that of enzyme-substrate interaction. Like enzymes, antibodies can reduce the activation energy required for chemical modification, upon stabilizing the transition state of the bound antigen. Keeping in mind the diversity of the CDRs in the antibody repertoire, it seems obvious that the antigen-binding domain of some antibodies may resemble that of an enzyme (Pauling, 1948). Such an antibody, upon interaction with its cognate antigen, may result in the chemical alteration of the antigen. In fact, antibody-mediated catalytic activity has been reported both in diseased individuals and upon immunization with suitable antigens (see next chapter).

2. Antibodies with catalytic activity

In the late 40's, a novel function of antibodies was postulated by Linus Pauling, which was later demonstrated experimentally: the capacity for antibodies to be endowed with enzymatic properties. Several strategies were developed to generate catalytic antibodies, or Abzymes, *a façon*, as will be explained below. The field has now evolved to the point where the use of "designed" catalytic antibodies may be applied to different disease conditions. The present chapter is dedicated to give a history of the research on catalytic antibodies, to describing the different approaches to generate antigen-specific catalytic antibodies, to discuss the physiopathological relevance of catalytic antibodies in human beings with a focus on antibodies with serine-protease like activity and, particularly, targeted towards human pro-coagulant factor VIII or human coagulation factor IX. Lastly, a description of the potential therapeutic usage of catalytic antibodies is summarized.

2.1. History and principles of antibody-mediated catalysis

The history of catalytic antibodies dates back to 1946 when Linus Pauling proposed the hypothesis that the active centre of an enzyme is targeted against the structure of the transition state, rather than to the native conformation of the substrate molecule (Pauling, 1948). He also proposed that if the antigen–binding domain of an antibody is generated randomly, then it is possible that the antigen–binding domain of some antibodies may possess structural similarity to an enzyme's active site and thus could stabilize the transition state of its substrate, similar to that of an enzyme. The hypothesis proposed by Pauling led Jencks in 1969, to propose that antibodies generated in an anti-hapten immune response against chemically stable analogs of the transition state of a reaction of

interest could potentially display an enzymatic activity (Jencks, 1969; Satterthwait and Jencks, 1974). Indeed, in 1985, a general method for generating catalytic monoclonal antibodies (mAbs) against transition-state analogs, and a way to use those antibodies to accelerate chemical reactions, was first described. In 1986, using this approach, Richard Lerner and Peter Schultz independently reported antibody catalysis for the first time: monoclonal antibody-mediated hydrolysis of aryl esters and of carbonates, respectively (Pollack et al., 1986; Tramontano et al., 1986). Since 1986, catalytic antibodies, catalyzing more than 100 distinct chemical reactions have been obtained using TSA. In the late 80's, the presence of spontaneously occurring catalytic antibodies in human beings opened new horizons and questions regarding their physiopathological relevance and the molecular and immune mechanisms that are at the basis of the generation of B-cell clones producing catalytic antibodies.

The principle of catalysis in abzymes is proposed to be mediated by the stabilization of the transition state of the substrate/antigen, which is then proceeded by breaking of chemical bonds. The catalytic activity in antibodies is conferred to the three dimensional spatial arrangement of amino acids, which together form either a catalytic triad (Sun et al., 1994) or dyad (Kolesnikov et al., 2000). Crystal structure of few abzymes with amidase activity, have shown to possess serine protease like catalytic triad (arrangement of serine, histidine and aspartate amino acids) with serine molecule acting as a nucleophile (electron-attacking group). In humans too, catalytic antibodies show the presence of serine protease like catalytic triad, as proposed by crystal structure of a catalytic monoclonal IgM obtained from a patient with monoclonal gammapathy (Ramsland et al., 2006). Indirect evidence for the presence of serine protease like

catalytic activity in human catalytic antibodies comes from their specific inhibition by serine protease inhibitors. However, serine protease like catalytic mechanism is not the whole sole mechanism of catalysis for all abzymes. An abzyme scFv derived from a semi-synthetic phage library with amidase activity, is known to posses tyrosine molecule as a nucleophile, which is very rare in nature (Reshetnyak et al., 2007). In addition, several monoclonal abzymes with similar catalytic function as their enzyme counterpart show less structural resemblance with each other and are known to posses' different amino acid configuration in the active site (Kolesnikov et al., 2000).

The location of amino acids participating in catalysis in an abzyme is not restricted to the antigen-binding site. Structure prediction of many monoclonal abzymes have shown that amino acids participating in catalysis are located in distinct regions of the variable domain, including the CDRs of heavy and light chains as well as the framework regions (Reshetnyak et al., 2007). While in some monoclonal abzymes, the amino acids participating in the formation of catalytic triad or dyad are contributed by close spatial arrangement of amino acids of both light and heavy chains, in others, either the light chain or the heavy chain solely contributes to the formation of an active site.

The catalytic efficiency of enzymes is a concerted effect of several individual properties, such as transition state stabilization, acid-base catalysis, proximity and strain. These properties together influence the rate of acceleration and make enzymes proficient catalysts. Antibodies provide an ideal scaffold for the installation of catalytic properties, however the exact replication of catalytic efficiency of enzymes in catalytic antibodies has not been achieved. This is due to the dissimilarities in the mechanisms by which enzymes and abzymes catalyze their substrate. A good example comes from the study of

the crystal structure of the aldolase antibody 33F12 (Barbas et al., 1997). Although both the abzyme 33F12 and its natural counterpart, fructose 1,6-diphosphate aldolase, function through a relatively simple catalytic mechanism involving a reactive lysine, there are marked differences in the sensitivity and specificity of the aldol reactions performed by them. Unlike natural aldolase, 33F12 performs nearly 100 different aldol condensation/addition reactions, however with a lower turnover rate. The crystal structure of the abzyme reveals that the reactive lysine is situated in a hydrophobic pocket that does not facilitate the entry of the substrate, thus lowering the turnover. In addition, the promiscuity of the binding pocket allows the abzyme to perform aldol condensation/addition on a wide variety of substrates. In a similar way, catalytic antibody 9A8 obtained by the anti-idiotypic approach, although possesses the catalytic dyad similar to its prototype enzyme acetyl- cholinesterase (AcChoE), displays relaxation in its specificity to substrates and inhibitors (Kolesnikov et al., 2000). The sequence analysis of 9A8 in comparison to AcChoE, revealed a striking difference in amino acids other than those involved in the catalysis. In the long run, the accumulation of data on the structures of abzymes will allow us to find the differentiating factors between enzymes and abzymes. This should enable generating tailor-made abzymes with fine sensitivity and specificity.

2.2. Strategies for deliberate induction of abzymes

2.2.1. Immunization with transition state analogs (TSA)

Making antibodies with binding pockets complementary to transition states is complicated by the fact that true transition states and most reaction intermediates are unstable. Thus, true transition states or intermediates cannot be isolated or used as haptens for immunization. Instead, so-called transition-state analog (TSA) molecules are used. Transition-state analog molecules are stable molecules that simply resemble a transition state (or intermediate) for a reaction of interest in terms of stereochemistry and charge distribution. To the extent that the transition-state analog molecule resembles a true reaction transition state or intermediate, the elicited antibodies will also be complementary to that transition state or intermediate and thus lead to the catalytic acceleration of that reaction (Figure 4A). Abzymes catalyzing the hydrolysis of amides and esters, as well as reactions of cyclization (Janda et al., 1993; Li et al., 1994; Wentworth et al., 1998), decarboxylation (Smiley and Benkovic, 1994; Barbas III et al., 1997; Hotta et al., 2000), lactonization (Napper et al., 1987), peroxidation (Ding et al., 1998), photochemical thymine dimer cleavage, bimolecular amide-bond formation, and other reactions not known to be catalyzed by natural enzymes have been described. Some abzymes have been produced that require cofactors for activity, similar to standard enzymes (Iverson and Lerner, 1989). Later, in 1995 a new concept of “reactive immunization” emerged, wherein organisms are immunized with highly reactive haptens designed to make covalent link with the nucleophilic B-cell receptor (BcR) on the B cells, thus forming a TSA (Wirsching et al., 1995). The catalytic efficiencies of aldolase

abzymes, generated by reactive immunization, are only 40-fold lower than most studied aldolases (Wagner et al., 1995; Barbas et al., 1997).

2.2.2. *Manipulation of the idiotypic network*

Generation of abzymes using the idiotypic network was inspired by the "idiotypic network theory" proposed by Niels Jerne in 1974 (Jerne, 1974). According to this theory, immunization of an animal with an antigen results in the production of a 1st generation antibody (Ab1), with an antigen-binding site specific for an epitope on the antigen. Immunization with the variable region of the Ab1 induces the production of 2nd generation antibodies (Ab2), the antigen-binding domain of which is complimentary to the variable domain of Ab1 and, in some cases, possesses the internal image of the antigen epitope. If the initial antigen is an enzyme, some of the Ab2s will present with an internal image of the catalytic site of the enzyme, and will thus be catalytic (Figure 4B). Manipulation of the idiotypic network has led to the generation of monoclonal abzymes with esterase activity (Izadyar et al., 1993), amidase activity (Avalle et al., 1998), carboxypeptidase activity (Hu et al., 1998), protease activity (Ponomarenko et al., 2007) and recently allinase activity (Li et al., 2012).

2.2.3. *Immunization with electrophilic covalent reactive analog (CRA)*

First reported in the year 2000, phosphonate diester molecules have been extensively used as a bait to fish-out antibodies with catalytic activity (Tramontano et al., 2000). Covalent reactive analogs are haptenic phosphonates that bind covalently to a nucleophilic residue in the active site of an abzyme or of the BcR of the abzyme-

producing B cells (Figure 4C). Haptenic phosphonates are reaction-based inhibitors, whose interaction in the active site of an abzyme is similar to that of a substrate. The only difference is, while a substrate molecule is released after conversion into product; the haptenic phosphonate makes a covalent linkage with the active site nucleophile. The interaction requires a nucleophilic attack by an amino acid onto the strongly electrophilic phosphonate in presence of a proton acceptor amino acid in close proximity. S. Paul and co-workers have obtained monoclonal abzymes against the gp120 coat protein of HIV-1 virus using CRA-coupled gp120 as an immunogen (Paul et al., 2003), and against the amyloid-β peptide by screening an scFv library against a CRA-derivatized amyloid-β peptide (Taguchi et al., 2008b). Likewise, Tramontano and colleagues have successfully used covalently reactive phosphonate esters to isolate catalytic scFv from a semi-synthetic scFv phage display library (Reshetnyak et al., 2007).

2.2.4. *Immunization with ground state antigens*

Immunization of normal or autoimmune-prone mice with ground state antigens has allowed to generate several monoclonal abzymes with catalytic activities against vasoactive intestinal peptide (Sun et al., 1994), CCR5 (Mitsuda et al., 2004), HIV-1 coat protein gp41 (Hifumi et al., 2002), gp120 (Durova et al., 2009), *H. pylori* urease (Hifumi et al., 2008) and TNFα (Hifumi et al., 2010). The group of Taizo Uda isolates abzymes among antigen specific binders by identifying the presence of serine protease-like catalytic triad by structure prediction of the gene sequences of heavy and light chain of antibodies.

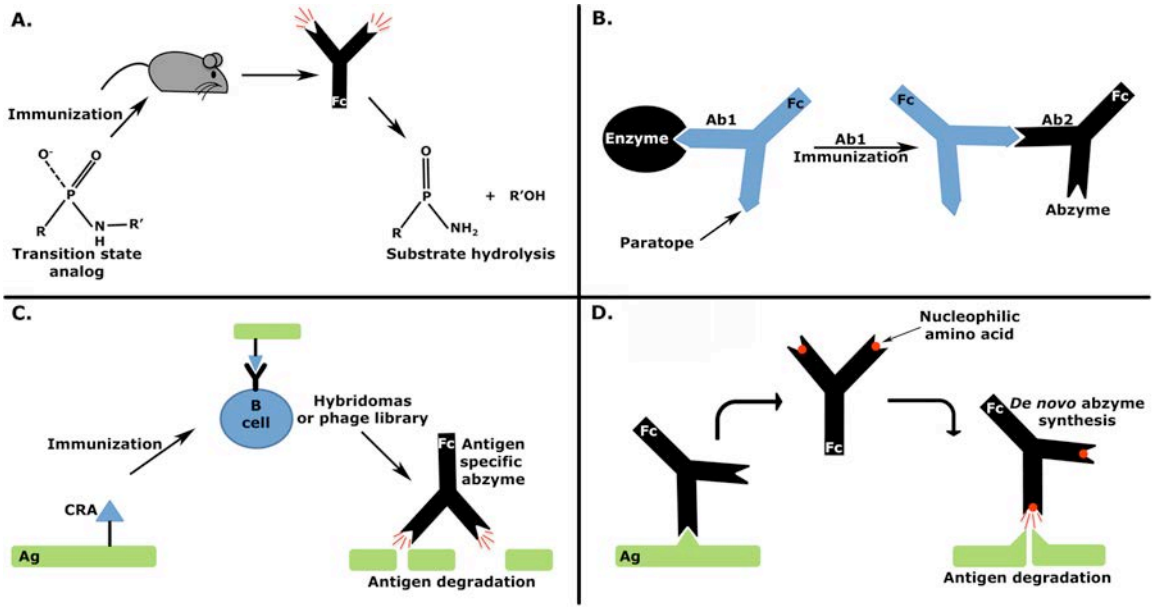
2.2.5. *De novo generation of catalytic antibodies by site-directed mutagenesis*

De novo elicitation of catalytic activity in antibodies has been performed by introduction of amino acid residues with known catalytic functions into the antigen-binding site of antibodies (Figure 4D). This approach is advantageous because the antibody, which is to be modified into an abzyme, is of a predetermined specificity. Fletcher et al, were able to introduce ribonuclease activity in an scFv variant of a Fab, which was specific for RNA. The authors targeted an Arg96 residue which was in close proximity of the 2'-OH of ribose sugar and replaced it with a histidine, in order to introduce a catalytic imidazole. The mutant scFv was able to hydrolyze RNA at a decent kinetic efficiency of 100 $M^{-1}sec^{-1}$ with the preservation of antigen specificity(Fletcher et al., 1998). In another instance, Liu et al were able to induce protease activity in a scFv specific for the bacterial protein HPr. Introduction of 4 mutations in the antigen-binding domain resulted in induction of protease activity with no loss in specificity for HPr. The authors introduced three catalytic amino acids; a glutamate to augment the nucleophilicity of a water molecule in the vicinity, a lysine to escalate the polarity of the carbonyl group and a histidine as a proton donor. A glycine moiety was added as a fourth mutation in order to stabilize the structural integrity of complementarity determining region (CDR H3)(Liu et al., 1998). In a similar approach, peptidase activity has been introduced into a light chain expressed at the yeast cell surface, by substituting two residues Ser27a and Asp1. The substitutions were done for the elicitation of a serine protease-like catalytic triad with a His93; already present within the CDR (Okochi et al., 2007).

2.2.6. *Isolation of catalytic antibodies by targeting specific germline genes*

Several abzymes possess variable region-encoding genes in the germline configuration (Le Minoux et al., 2012). In line with this observation, the presence of the Asp1, Ser27a and His93 catalytic triad was investigated in mouse kappa light chain of a total of 84 clones by the group of Uda: pseudo catalytic triads were found mainly in the bb1, cr1, cs1, bl1, bj2 and bd2 germline kappa genes (Uda and Hifumi, 2004). In parallel, the same group screened genes encoding human kappa light chains, upon structural prediction using a strict spatial arrangement of the catalytic residues Ser, His and Asp; distance between His-Ser to be within 10 angstrom and that of His-Asp within 15 angstrom. Nine germline genes belonging to subgroup II showed a high prevalence of pseudo catalytic triads. Cloning and expression of these genes from circulating B cells of a healthy individual vaccinated against rabies virus, allowed the generation of virus-specific abzymes (Hifumi et al., 2012).

Figure 4.



2.3. Catalytic antibodies under physiological conditions

As explained above, the immune system is equipped with an incredible machinery of generating diversity. While catalytic antibodies may be induced using appropriate immunogens, as explained above, it has now become clear that the immune system is able to spontaneously generate antibodies with catalytic activity, both in health and disease.

Catalytic antibodies are now considered to be a part of the natural antibody repertoire and act as defense against bacterial and viral pathogens. In fact, catalytic antibodies of the IgA isotype with anti-bacterial/viral functions have been obtained from human saliva and from the milk of healthy mothers (Semenov et al., 1998; Mitsuda et al., 2007). A set of

evidence shows that natural catalytic antibodies with promiscuous antigen-binding are encoded by the germline genes (Gololobov et al., 1999). Immunoglobulins M are considered to be the closest to the germline and data on polyclonal IgM from naïve mice and healthy humans show promiscuous catalytic activity of IgM against synthetic peptide substrates (Planque et al., 2004). Additionally, catalytic antibodies derived by deliberated immunization are also known to posses' germline configuration (Kolesnikov et al., 2000). Further validation of the germline origin of catalytic antibodies has been done, by performing site-directed mutagenesis of a catalytic antibody light chain (Gao et al., 1995). Upon replacing the amino acids that were introduced by somatic hypermutation with the germline counterpart, the catalytic activity was not abrogated. Additional information regarding the germline origin of catalytic antibodies comes from the study of Le Minoux et al., analysis of the gene sequence of 23 monoclonal catalytic antibodies with amidase activity, revealed more than 97% resemblance in the heavy and light chain genes with the germline counterpart (Le Minoux et al., 2012). This suggests that antibody mediated catalysis is an innate property of the immune system, which is encrypted in the germline. The presence of antigen-specificity however, is absent in naturally occurring abzymes. Among naturally occurring abzymes in healthy individuals, IgM are known to show the highest catalytic efficiency, followed by IgA and least in IgG. The μ-chains of the splenic B cells are known to be the most abundant proteins that possess nucleophilic sites; identified by the binding of labelled electrophillic phosphonate inhibitors. Finally, naturally occurring catalytic antibodies are known to behave like serine proteases by virtue of their specificity of cleavage at the basic amino acid residues.

2.4. Catalytic antibodies under pathological conditions

Experimental data on autoimmune-prone MRL/lpr or SJL mice shows that the frequency of catalytic clones is much higher in these strains as compared to normal mice (Tawfik et al., 1995). Accordingly, in 2000, the work from the group of Alexander Gabibov showed a higher frequency of abzymes with protease and nuclease activity in strains of autoimmune prone mice, MRL-lpr/lpr, NZB/NZW, SJL/J, in comparison to normal Balb/C mice (Ponomarenko et al., 2000).

In humans too, under several autoimmune and inflammatory conditions, including asthma (Paul et al., 1989), acquired hemophilia A (AHA) (Wootla et al., 2008a), systemic lupus erythematosus (SLE) (Shuster et al., 1992), Hashimoto's thyroiditis (HT) (Li et al., 1995), scleroderma (Bronshtein et al., 1992), multiple sclerosis (MS) (Ponomarenko et al., 2006), Alzheimer's disease (AD) (Taguchi et al., 2008a), a high level of abzyme activity is witnessed against self-antigens (Table 1). Thus, a high prevalence of DNA/RNA-hydrolyzing antibodies has been found in patients with SLE, scleroderma and MS. In addition, IgG from patients with MS also show hydrolysis of myelin basic protein. Li et al reported the presence of thyroglobulin (Tg)-cleaving antibodies in serum of patients with Hashimoto's thyroiditis. The presence of Tg-cleaving antibodies in HT patients was hypothesized to be beneficial in clearing-up of the circulating Tg, which could otherwise feed in the immune response. Patients with Alzheimer's disease were reported to have amyloid beta (Aβ)-cleaving IgM antibodies. It has been proposed that the deposition of Aβ in the brain induces neurodegenerative disorders. The presence of Aβ-hydrolyzing antibodies may thus provide a feasible

mechanism of Aβ clearance from the body. Acquired hemophilia A is a serious autoimmune disease marked by the sudden appearance of autoantibodies against coagulation factor VIII (FVIII). Autoantibodies against FVIII prevent its interaction with other molecules of the coagulation cascade thereby inhibiting its procoagulant activity. In a previous study, nearly 50% of the studied patients with anti-FVIII autoantibodies in AHA also showed the presence of FVIII-hydrolyzing IgG. The FVIII-hydrolyzing activity of IgG from few of these patients was also found to co-evolve with the inhibitory titre, suggesting that FVIII-hydrolyzing antibodies may participate to the FVIII inactivation process.

In all the above-mentioned studies, the level of catalytic antibodies to the respective disease-specific antigen were either extremely low or absent in the serum of normal control subjects. Based on these findings, it was tempting to speculate that the adaptive improvement of innate catalysis against self-antigens is a disfavoured event in physiology and occurs only when there is a breakdown in the mechanisms of self-tolerance.

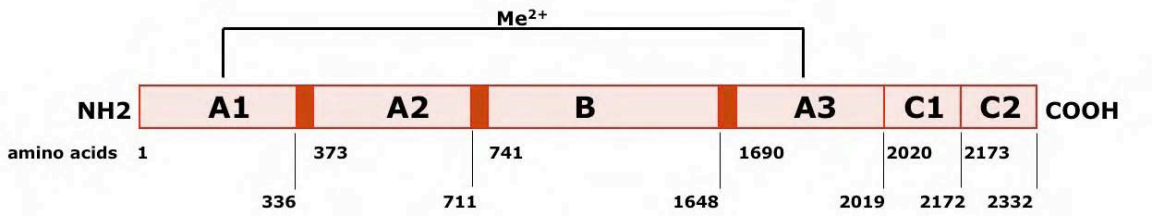
Table 1. Catalytic antibodies in various pathological conditions

Pathology	Target antigen
Inflammatory disorders	
Asthma	Vasoactive intestinal peptide (VIP)
Sepsis	FVIII, FIX
Autoimmune disorders	
Hashimoto's thyridoitis	Thyroglobulin
Systemic lupus erythematosus	DNA, RNA
Scleroderma	DNA, RNA
Rheumatoid arthritis	DNA, RNA
Multiple sclerosis	DNA, RNA, MBP
Acquired hemophilia	FVIII, FIX
Alzheimer's disease	Aß-peptide
Metabolic disorder	
Diabetes	Grp94
Infectious disorder	
Immune thrombocytopenia associated with HIV-1 infection	Platelet GPIIIa
Neoplastic disorder	
Multiple myeloma	Prothrombin
Alloimmune disorders	
Hemophilia A	FVIII
Renal transplant rejection	FVIII, FIX

2.4.1 Catalytic antibodies against coagulation factors

Past work in the host laboratory has documented the presence of catalytic antibodies able to hydrolyse several coagulation factors: factor VIII and factor IX. The underlying hypothesis was that coagulation factor-hydrolyzing IgG may modulate the thrombotic profile of the patients, and thus have an outcome on disease progression. In the course of my PHD work, I have concentrated on two pathological situations in the human wherein either bleeding or thrombotic conditions are implicated: acquired hemophilia and rejection of renal transplant.

Figure 5. Structure of FVIII: Factor VIII is released by the hepatocytes as a heterodimer of heavy chain (domains A1-A2-B) and a light chain (domains A3-C1-C2). A non-covalent divalent metal ion linkage between A1 and A3 domains associates the heavy and light chains.



2.4.2 Catalytic antibodies in patients with acquired hemophilia A

Acquired hemophilia A (AHA) is a bleeding disorder that occurs following the development of a neutralizing autoimmune response against the endogenous coagulation factor VIII (FVIII). AHA occurs at an overall incidence rate of 1.48 per million/year and it mainly affects the elderly, with an incidence rate as high as 14.7 per million/year seen in patients above 85 years (Collins et al., 2007). The clinical feature of AHA is the occurrence of sudden bleeding episodes in individuals without previous bleeding history. Bleeding is life threatening in about 80% of the cases, especially in the early stage of the disease or in cases of excessive bleeding following trauma, surgery or cerebral hemorrhages (Collins et al., 2007). The bleeding patterns in AHA include a high prevalence of hemorrhages in skin, muscle or soft tissue and mucous membrane (Green and Lechner, 1981; Boggio and Green, 2001).

In our seminal work, we had demonstrated the presence of IgG with proteolytic activity towards exogenous therapeutic FVIII in inhibitor-positive patients with severe congenital hemophilia A (Lacroix-Desmazes et al., 1999). FVIII-hydrolyzing IgGs was detected in about 50% of the patients included in the study (Lacroix-Desmazes et al., 2002). Further, the rates of IgG-mediated FVIII proteolysis measured *in vitro* were positively correlated to the inhibitory activity measured in the plasma of the patients (Lacroix-Desmazes et al., 2002), although it is obvious that the different anti-FVIII IgG in the polyclonal antibody population of a single patient may simultaneously neutralize therapeutic FVIII by several means: classical steric hindrance and proteolysis. Importantly, the proteolysis of FVIII by IgG was demonstrated to lead to FVIII inactivation *in vitro* (Lacroix-Desmazes et al., 2006). The protease activity was similar

to that of serine proteases, as more than 80% of the identified cleavage sites on FVIII occurred after a lysine or an arginine (Lacroix-Desmazes et al., 2006).

In the case of AHA, we observed that IgG from about 47% of the patients included in our cohort also hydrolyzed FVIII (Wootla et al., 2008a). A longitudinal study, performed on four AHA patients to evaluate the relationship between FVIII hydrolysis and FVIII inhibition, suggested a co-evolution of the catalytic activity with the inhibitory titer over time. However, despite the inhibitory potential of FVIII-hydrolyzing IgG in AHA patients, no correlation was found between the presence/absence of proteolytic IgG and the clinical parameters of the patients, or the survival status of the patients 1 year after diagnosis. The results of this study did not enable us to attribute the epitope specificity or the type I/II kinetics of FVIII inhibition to the presence of FVIII-hydrolysing IgG.

The hydrolysis of coagulation factor VIII (FVIII) by IgG purified from the serum of patients with congenital and acquired hemophilia A (Lacroix-Desmazes et al., 1999; Lacroix-Desmazes et al., 2002; Lacroix-Desmazes et al., 2006; Wootla et al., 2008a; Wootla et al., 2009), suggests that catalytic antibodies may interfere with hemostasis under pathological conditions. Interestingly, IgG-mediated FVIII and factor (FIX) hydrolysis had already been documented in patients who survived septic shock (Lacroix-Desmazes et al., 2005), with a potential of modulating disseminated vascular thrombosis. Furthermore, we found that IgGs from some AHA patients too were able to hydrolyze FIX, resulting in its activation. Hence we envisaged identifying the relevance of this phenomenon in the outcome of AHA. The details of this observation are attached as a publication in the result section.

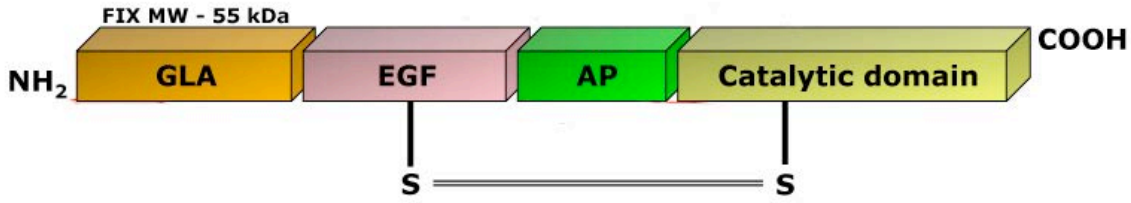
2.4.3 Catalytic antibodies in patients with chronic allograft nephropathy

Chronic allograft nephropathy (CAN) is one of the main causes of late renal allograft rejection. It is characterized by loss of function of the graft due to tissue destruction, which is substantially caused by the induction of the coagulation cascade by the stressed endothelium of the graft.

The deleterious role of catalytic antibodies has been suspected in other pathological situations, like hemophilia, multiple sclerosis (Ponomarenko et al., 2006) and HIV-1-related immune thrombocytopenia (Nardi et al., 2001). Under pathological conditions as hemophilia, where the presence of coagulation FVIII is indispensible, the presence of FVIII-hydrolyzing IgG is expected to be deleterious. However, under pathological conditions such as sepsis and CAN, which are characterized by uncontrolled activation of coagulation, the presence of coagulation factor-hydrolyzing antibodies, could play a beneficial role. In a previous retrospective study, we obtained serum at two different time intervals (3 months and 2 years post-transplantation) from patients that had undergone renal graft transplant (Wootla et al., 2008b). Some patients in the cohort were known to develop chronic allograft nephropathy (CAN) following renal transplantation. A surrogate synthetic peptide chromogenic substrate PFR-MCA was used to screen IgG-mediated catalytic activity. Interestingly, IgG from patients without CAN, demonstrated significantly higher catalytic activity as compared to that from patients with CAN, both 3 months and 2 years post-transplantation. In addition, the catalytic activity measured at 3 months was predictive of CAN that developed 2 years later. The purified IgG from some of the patients included in the study were able to hydrolyze the coagulation factors VIII and/or IX. Also, IgG-mediated catalytic activity against synthetic substrate correlated

with FVIII hydrolysis. Thus, we postulated that the presence of catalytic IgG in CAN patients could be used as a prognostic marker to predict favorable outcome in renal graft transplant. However, the observations needed further validation in a prospective study with a larger cohort of patients. We designed a prospective study on 100 consecutive renal transplant patients and the observations are mentioned in the results section.

Figure 6. Structure of FIX: Factor IX is a vitamin K dependent coagulations factor secreted by the liver. The light chain of FIX is constituted of γ-carboxyglutamic acid residues (GLA) and a domain homologous to epidermal growth factor (EGF). The light chain is attached to the heavy chain through an activation peptide (AP) region, which is cleaved from the FIX upon activation. The heavy chain constitutes the catalytic domain of FIX.



2.5. Therapeutic potential of catalytic antibodies

The antibody scaffold provides a unique combination of antigen specificity and functional activity to generate novel enzymes. Nowadays, attempts are made to take advantage of these synergistic properties for the treatment of human diseases.

2.5.1 Catalytic antibodies against infectious agents

Several virus-specific catalytic antibodies have been developed in the recent years. Thus, the HIV-1 coat proteins gp120 and gp41 have been targeted because of the key role they play in infecting CD4+ T lymphocytes. Three monoclonal abzymes able to cleave gp120 in a site-specific manner were generated upon immunization of mice with a CRA-coupled gp120 (Paul et al., 2003). Because HIV-1 is known to escape from the host immune system by undergoing repeated mutations in its coat proteins, abzymes were also generated from a phage library established from lupus patients that cleaved the conserved superantigenic region 421-433 (Nishiyama et al., 2007). In parallel, the group of Uda has generated abzyme light chains by ground state immunization of the conserved region of gp41 (Hifumi et al., 2002).

Catalytic kappa light chains have been isolated from an individual vaccinated against rabies virus; the light chains cleaved small peptide substrates and, in some cases, diminished the infectivity of rabies virus (CVS-11) in mouse NA cells and protected mice upon intracerebral inoculation with CVS-11 (Hifumi et al., 2012).

Lastly, the group of Uda has isolated a light chain abzyme (UA15-L) specific for the urease of *H. pylori* (Hifumi et al., 2008). UA15-L was able to hydrolyze both

recombinant UreB and the whole urease, and degraded urease in the intact bacterium, thus significantly decreasing colonization by *H*. pylori in the stomach of mice.

2.5.2 Abzyme prodrug therapy

The first documentation of abzyme-mediated activation of a prodrug was provided in 1993, when Miyashita et al showed the activation of a non-bioactive chloramphenicol ester by an abzyme generated against a phosphonate TSA (Miyashita et al., 1993). Later in 1996, Wentworth et al generated an abzyme that activated the carbamate prodrug into its cytotoxic form, nitrogen mustard, that was able to decrease the viability of human colonic carcinoma cells *in vitro* (Wentworth et al., 1996). The most studied abzyme for prodrug therapy is 38C2 that was generated by reactive immunization with the 1,3 diketone hapten, and possesses an aldolase activity (Wagner et al., 1995). 38C2 activates the prodrug form of the anti-cancer drugs doxorubicin and camptothecin (Shabat et al., 1999). It was shown to inhibit the growth of human colon carcinoma and prostrate cancer cell lines and to reduce tumor growth in an animal model of neuroblastoma (Shabat et al., 2001). 38C2 also showed therapeutic potential in diabetes upon activation of an aldol-modified insulin (Worrall et al., 2001). More recently, a catalytic nanobody with alliinase activity was obtained upon manipulation of the idiotypic network. The abzyme nanobody suppressed the growth of the B16 tumor cell line by converting the precursor prodrug alliin into cytotoxic allicin (Li et al., 2012).

2.5.3 Catalytic antibodies in inflammation

A potential beneficial role of catalytic antibodies has been investigated in Alzheimer's disease by virtue of their capacity to proteolytically degrade amyloid aggregates. Proteolytic cleavage of amyloid-β by recombinant light chains (Rangan et al., 2003), heterodimeric light chains and single chain abzymes (Taguchi et al., 2008b) was documented with exceptionally elevated hydrolysis rates. It was proposed that single chain variant abzymes may combine efficient Aβ clearance and prevention of Fc-mediated inflammation caused by phagocytic cells.

A monomeric heavy chain abzyme specific for the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) was recently developed upon immunization with the ground state molecule (Uda and Hifumi, 2004). In the context of the treatment of autoimmune and inflammatory diseases by anti-TNF therapy (Suryaprasad and Prindiville, 2003; Lamprecht, 2005; Atzeni et al., 2009; Rosenblum and Amital, 2011), the capacity of abzymes to turnover should allow to diminish the requirement for bolus doses of therapeutic antibodies that are usually required for the clearance of TNF-α.

2.5.4 Catalytic antibodies for gene silencing

Gene silencing by targeting mRNA has been achieved by conventional small interfering RNA (siRNA) technique. Alternatively, inhibition of intracellular proteins by cell penetrating antibodies (TransMabs) is also being investigated (Muller et al., 2005). In a novel approach, Lee et al have achieved gene silencing by catalytic antibodies specific for the mRNA of Her2, a protein that is overexpressed in breast cancer cells (Lee et al., 2010). The abzymes are variants of a DNA/RNA-hydrolyzing antibody light chain 3D8,

obtained by generating a synthetic library by randomization of amino acids. The catalytic antibodies variants selectively hydrolyzed the Her2 mRNA, resulting in decreased surface expression of Her2 and induction of apoptosis, with a cytotoxic potential greater than that of siRNA.

3. Polyreactive antibodies

According to the clonal selection theory proposed by Burnet and Medawar in the 1950's, single antibody molecules are specific for a single antigen. It has however become clear over the last 3 decades that some monoclonal antibody molecules are able to engage interactions with different structurally unrelated antigens, a property coined as "polyreactivity". The broad binding specificity of polyreactive antibodies is conferred by low affinity and conformational flexibility of the antigen-binding domain. From an evolutionary point of view, polyreactivity in antibodies are considered to be a conserved feature of the immune system and are found in distantly related species, as in humans and sharks (Marchalonis et al., 2001). Thus, the repertoire of antibodies that circulates in a single healthy individual is composed of a substantial part of "natural" polyreactive antibodies. In addition to antibodies that are intrinsically polyreactive, our group and others have demonstrated that exposure of some monoreactive antibodies to different protein destabilizing agents may also impart polyreactivity to the antibodies, a phenomenon referred to as "induced" or "cryptic" polyreactivity and that may be considered as an additional level of generation of antibody-binding diversity (Dimitrov et al., 2006; Dimitrov et al., 2007; Djoumerska-Alexieva et al., 2009; Djoumerska-Alexieva et al., 2010). This part of the introduction is dedicated to summarizing our present knowledge on "natural" and "induced" polyreactive antibodies and lays the ground to the question I have addressed in the course of my PhD.

3.1. Natural polyreactive antibodies

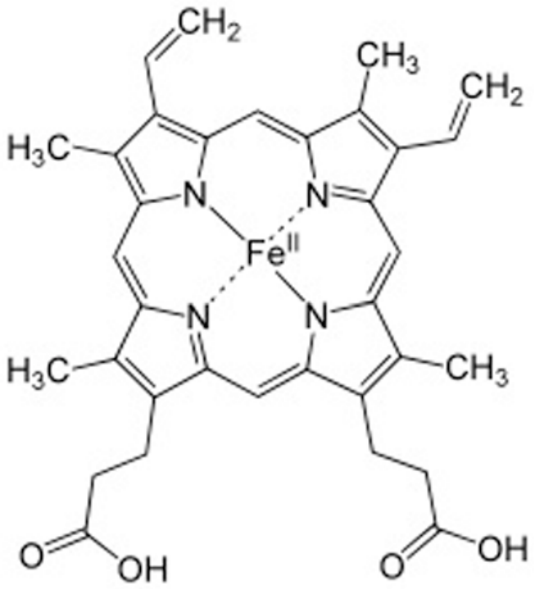
Naturally occurring polyreactive antibodies are believed to be generated in the absence of any known antigenic stimulation. Early precursor B cells are considered to be the major producers of polyreactive antibodies (Wardemann et al., 2003). In newly born children, 50% of the cord blood B cells exhibit the presence of polyreactive BCRs (Chen et al., 1998). However, in adults the percentage of polyreactive B cells are between 15-20%. B cells that produce polyreactive antibodies are widely distributed in the lymphoid organs and the repertoire consists of both the B-1^+ and B-1^- lymphocytes (Chen et al., 1998; Wang et al., 2001).

While monoreactive antibodies bind to their cognate antigen following the rigid "lock and key" mechanism, the binding of natural polyreactive antibodies to antigens is less rigid and allows conformational changes within the binding pocket. Polyreactive antibodies have germline or near germline configuration of the variable domain. The near germline configuration provides greater flexibility of interactions with different antigens. Among the different isotypes, IgM are considered to be the most polyreactive due to close similarity with the germline, although some IgA and IgG also show polyreactivity. Another important aspect of polyreactive antibodies is their low antigen binding affinity ($K_D = 10^{-4} – 10^{-7}$ M) pertaining to low somatic mutations, as compared to monoreactive antibodies (Ternynck and Avrameas, 1986; Burastero et al., 1988; Nakamura et al., 1988; Casali and Notkins, 1989). The role of CDRs in conferring polyreactivity has been proved by experiments showing maintenance of polyreactivity in the Fab form, persistence of polyreactivity after implantation of a polyreactive CDR onto a

monoreactive antibody scaffold and abrogation of polyreactivity by site-directed mutagenesis of CDRs. However, no consensus has been observed in the composition of amino acids in the CDRs of polyreactive antibodies (Notkins, 2004). It is also well accepted that the primary structure of the heavy or light chain is responsible in promoting polyreactivity of a CDR (Harindranath et al., 1993). This view is also backed by the observations that antibodies can exist in different isoforms even without binding to the antigen (Foote and Milstein, 1994) and that upon interaction with antigen, conformational changes may allow accommodation of the antigens (James et al., 2003).

Natural polyreactive antibodies are considered to play an important role in the broad anti-bacterial activity of the normal serum. In fact, *in vitro* studies show that natural polyreactive antibodies and B-cells expressing natural polyreactive antibodies bind to a wide range of self and bacterial/viral antigens (Zhou et al., 2007). Thus polyreactive antibodies may play a role in preventing the dissemination of pathogens by sequestering them to the lymphoid organs. Moreover, the role of polyreactive antibodies in mucosal immunity can be pointed out by the presence of polyreactive IgA antibodies in salivary secretion and colostrum (Quan et al., 1997). Due to low affinity binding with the antigens the polyreactive B cells are not able to present co-stimulatory molecules B7-1 and B7-2; however they are able to process the antigens (Wang et al., 2001). By virtue of this property polyreactive B cells may play a role in maintaining the peripheral immunological tolerance.

Figure 7. Structure of heme:



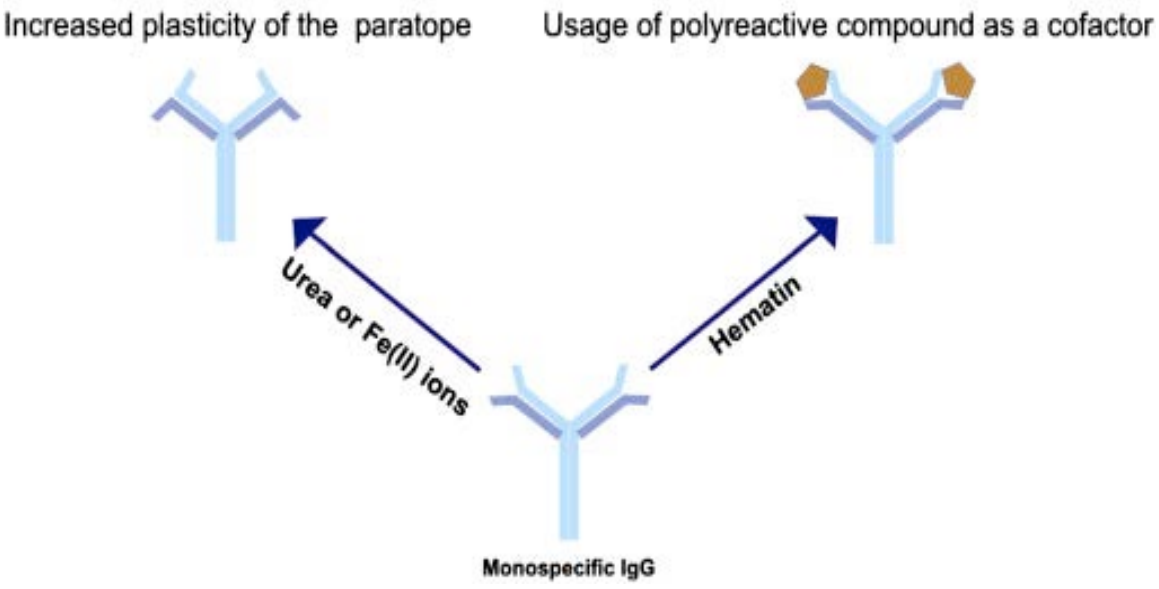
3.2. Induced polyreactive antibodies

In contrast to natural polyreactive antibodies, induced polyreactive antibodies are inherently monoreactive; they become polyreactive upon treatment with protein destabilizing factors, such as chaotropic agents, low and high pH, high-salt concentration, elevated temperature, or oxidative agents.

Post-translational modifications of monospecific antibodies with protein destabilizing agents lead to conformational changes in the antibody paratopes, making them sensitive to form novel interactions (Bouvet et al., 2001). Thus, the treatment of antibodies with urea or ferrous ions leads to the change in the thermodynamics of the antigen-antibody interactions (Dimitrov et al., 2006; Dimitrov et al., 2007). *In vitro* studies have shown that although the affinity of antigen recognition remains the same, there is a prolongation in the association and dissociation kinetics, which indicates an increase in the flexiblity of the paratopes. The induction of plasticity in antigen recognition is considered to be due to

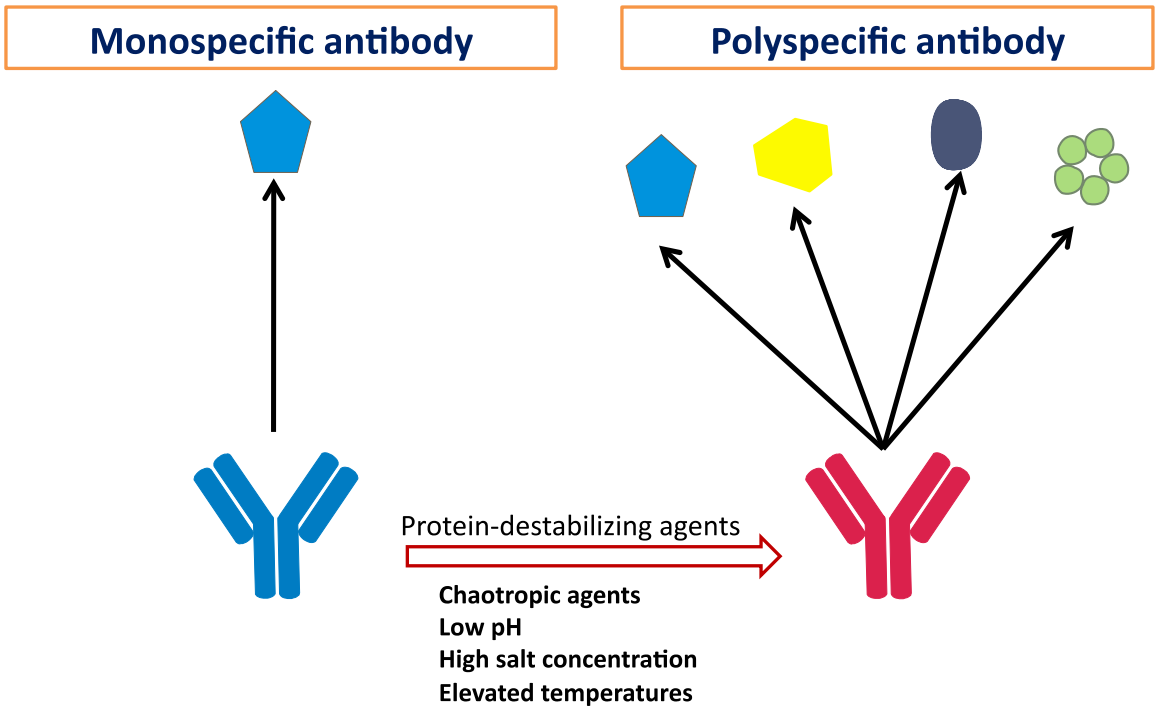
the partial denaturation of the variable regions. Among all the inducers of polyreactivity, heme shows a distinct mechanism of inducing polyreactivity. Unlike other inducers, heme does not increase the flexibility, rather makes the variable domain more rigid (Dimitrov et al., 2007) (Dumont et al., 1994). However, heme by itself establishes several non-covalent interactions (hydrophobic, hydrogen bonds, van der Waals, ionic and coordinative-interactions), by virtue of its hydrophobic macrocyclic structure (Figure 7, 8).

Figure 8. Different mechanisms of induction of polyreactivity in mono-reactive antibodies by protein-destabilizing agents:



Sites of inflammation are a rich source of such protein destabilizing agents. *In vivo*, the sources of such agents are immune cells like phagocytes, which create a pro-oxidative environment by the release of reactive oxygen species (ROS). In addition, inflammatory sites are rich in pro-oxidative labile-iron pool and free iron containing cofactors (heme) that can induce polyreactivity in antibodies. Under diseased conditions such as hemolysis, ischemia-reperfusion, or hemorrhages, large quantities of hemoproteins are released, which, upon oxidation, easily release their prosthetic groups once they are outside the cells. The excessive release of heme saturates the heme-scavenging proteins and the amount of free heme can reach up to 20 μM in plasma (Balla et al., 2003). Immunoglobulins, that are one of the most abundant plasma proteins, may bind the free heme and acquire polyreactivity. In fact, heme-exposed immunoglobulins show reactivity with bacterial antigens as well as whole bacteria and show enhanced bacterial killing via initiation of complement cascade (Dimitrov et al., 2007). *In vivo* experiments on mouse model of sepsis have also shown protective effect of IVIg modified with protein-destabilizing agents (Bouvet et al., 2001; Dimitrov et al., 2006). In addition, modified IVIg have also shown beneficial effect in treated animals by lowering the plasma levels of pro-inflammatory mediators (unpublished data). Thus, induced polyreactivity may serve as buffering agents of the immune system. However, similar observations were not seen in the case of pooled normal IgM (IVIgM). Upon treatment with ferrous ions, IVIgM did not show pronounced antigen-binding capacity (Dimitrov et al., 2006).

Figure 9.


Monospecific antibody
Polyspecific antibody
Protein-destabilizing agents
Chaotropic agents
Low pH
High salt concentration
Elevated temperatures

4. Objectives of the present study

In the introduction part of the thesis, I have presented the basic knowledge on catalytic antibodies, their beneficial/deleterious effect in various diseases, various methods to generate monoclonal catalytic antibodies and their scope as therapeutics. I have also presented in brief our understanding on the physiological relevance of natural polyreactive antibodies and mechanism of induction of polyreactivity by protein destabilizing agents. Based on this understanding, my main objective was to understand the pathophysiological relevance of catalytic antibodies in diseased conditions such as acquired hemophilia and renal transplant. Further, I also attempted to better decipher the mechanism underlying the induction of polyreactivity in antibodies.

Objective 1. Description of FIX-hydrolyzing antibodies in acquired hemophilia A

Our group has previously demonstrated the presence of catalytic antibodies against FVIII in patients with acquired hemophilia A (AHA). During our investigation, we observed FIX hydrolysis by IgG of some patients. The hydrolysis product was suspected to be the activated form of FIX. In this work, we investigated whether catalytic IgG from AHA patients are able to activate human FIX *in vitro*, whether IgG-mediated FIX activation has a relevance in the outcome of AHA.

Objective 2. Investigate the evolution of catalytic antibodies in renal transplant

Catalytic antibodies have been described in diverse autoimmune and inflammatory conditions. However, the physiopathological relevance of catalytic antibodies remains unclear. While under most disease conditions, the presence of catalytic antibodies is

related to deleterious consequences, in some diseases such as sepsis, their increased prevalence has been associated to favourable disease outcome. We have previously demonstrated in a small cohort of patients with CAN, a positive association between higher levels of catalytic IgG and renal graft survival. I decided to revisit the phenomenon in a larger cohort of patients in a prospective study. My assumption was that the establishment of a large cohort of patients followed over several years would provide valuable information regarding the evolution of IgG-mediated catalytic activity under disease condition.

Objective 3. Investigate the role of antibody constant domain in heme-induced polyreactivity

A relatively large portion of the natural antibody repertoire is known to be polyreactive. Apart from the naturally occurring polyreactive antibodies, some antibodies can be induced to display polyreactivity by protein destabilizing agents. While most of the protein destabilizing agents induce polyreactivity by making the variable domain flexible, an iron-cofactor, heme is known to make the variable domain rigid. Interestingly, while some antibodies are susceptible to heme-induced polyreactivity, others are not. In addition, the importance of the nature of constant domain in conferring sensitivity to heme-induced polyreactivity has not investigated. The investigation of the role of constant domain in heme-induced polyreactivity may provide greater insight into the possible mechanisms by which a heme induces polyreactivity.

Results

Article 1- Résumé en Français

Titre: Activation du facteur IX par les anticorps protéolytiques chez les patients atteints d'hémophilie acquise

L'hémophilie acquise est une maladie hémorragique rare qui est caractérisée par l'apparition spontanée d'anticorps inhibiteurs dirigés contre le facteur VIII (FVIII). Les IgG de certains patients atteints d'hémophilie acquise hydrolysent le FVIII. En raison de l'étiologie complexe de la maladie, aucun paramètre clinique, y compris la présence d'IgG hydrolysant le FVIII, ne permet de prédire la survie ou le décès des patients. Ici, nous démontrons la présence d'anticorps anti-facteur IX (FIX) chez les patients avec hémophilie acquise. Les IgG de certains patients hydrolysent le FIX. Dans la plupart des cas, l'hydrolyse du FIX par les IgG résultait dans l'activation du FIX. Ainsi, l'hydrolyse du FIX par les IgG conduisant à une génération significative de FIX activé chez 25 des 65 patients inclus dans l'étude. Sur la base de notre estimation des paramètres cinétiques, les IgG des patients activaient jusqu'à 0,3 nM de FIX par 24 heures, une quantité capable de restaurer le génération de thrombine *in vitro*, à condition que ≥3% de FVIII résiduel soient disponibles dans le plasma. Notre travail identifie les IgG protéolytiques comme de nouveaux activateurs du FIX dans les conditions pathologiques. L'activation du FIX par les IgG apparaît comme un phénomène prévalent au sein d'une population de patients avec hémophilie acquise. La présence de telles IgG pourrait compenser au moins en partie l'inhibition du FVIII endogène par les autoanticorps et rétablir une capacité de génération de thrombine.

Proteolytic antibodies activate factor IX in patients with acquired hemophilia

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Acquired hemophilia is a rare bleeding disorder characterized by the spontaneous occurrence of inhibitory antibodies against endogenous factor VIII (FVIII). IgG from some patients with acquired hemophilia hydrolyze FVIII. Because of the complex etiology of the disease, no clinical parameter, including the presence of FVIII-hydrolyzing IgG, has been associated with patient's survival or death. Here, we demonstrate the presence of anti-FIX antibodies in acquired hemophilia patients. IgG from some patients were found to hydrolyze FIX. In most cases, IgG-mediated FIX-hydrolysis resulted in FIX activation. IgG-mediated hydrolysis of FIX thus led to the significant generation of activated FIX in 25 of 65 patients. Based on the estimated kinetic parameters, patients' IgG activated up to 0.3nM FIX in 24 hours, an amount that restored thrombin generation in vitro provided the presence of more than or equal to 3% residual FVIII activity in plasma. This work identifies proteolytic IgG as novel molecules able to activate FIX under pathologic conditions. IgG-mediated FIX activation is a prevalent phenomenon among acquired hemophilia patients. The presence of FIX-activating IgG may partly compensate for the antibody-mediated inhibition of endogenous FVIII in restoring thrombin generation. This clinical trial was registered at www.clinicaltrials.gov as #NCT00213473. (*Blood*. 2011;117(7):2257-2264)

Introduction

Acquired hemophilia is a severe hemorrhagic autoimmune disorder that occurs in approximately 1 per 1 million persons each year. It is characterized by the spontaneous development of autoantibodies directed against endogenous factor VIII (FVIII), the cofactor of activated FIX in the coagulation cascade. Clinical features include bleeding in mucosal and soft tissues, hematuria, hematemesis, or melena and prolonged postpartum or postoperative bleeding.[1] In most patients, anti-FVIII autoantibodies are idiopathic. However, the disorder is associated with other conditions in approximately 40% to 50% of cases, which mainly occur in relation to postpartum, autoimmune diseases, malignancies, and drug administration.[2] The reported mortality is between 6.2% and 44.3% one year after diagnosis.[3]

Anti-FVIII autoantibodies, also referred to as FVIII inhibitors, neutralize FVIII procoagulant activity by steric hindrance, thus preventing the interaction of FVIII with activated FIX, von Willebrand factor, phospholipids, thrombin, and FX.[4] By binding to FVIII, anti-FVIII antibodies may also accelerate the clearance of FVIII. In addition to these mechanisms, IgG from some patients with acquired hemophilia hydrolyze FVIII.[5] Because of the complex etiology of the disease, no clinical parameter, including the presence of FVIII-hydrolyzing IgG, is known to predict the outcome of the disease.

FIX inhibitors have occasionally been reported in patients with acquired hemophilia, alone[6-10] or in combination with FVIII inhibitors.[11,12] Here, we investigated the prevalence of proteolytic IgG against FIX among patients with acquired hemophilia.

Methods

Patients

Frozen plasma samples from 65 patients with acquired hemophilia were obtained at the time of diagnosis from Centre Hospitalier Universitaire de Rouen (Etude Sacha: 41 patients), Centre Hospitalier Universitaire de Caen (11 patients), Hôpital Cochin (Paris), Hôpitaux du Kremlin-Bicêtre (Bicêtre), Nîmes, and Centre Hospitalier Universitaire de Compiègne (France: 13 patients), in accordance with the local ethical regulation and with informed consent in compliance with the Declaration of Helsinki. The study protocol has been approved by the ethics and data protection committees (Comité Consultatifs de Protection des Personnes se prêtant à des Recherches Biomédicales, Commission Nationale de l'information et des Libertes [CCPPRB, CNIL]). "Etude Sacha" includes clinical data from 82 patients with acquired hemophilia collected in France.[13] 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 FVIII activity less than 30% (FVIII $> 30\%$ in the case of 2 patients), an inhibitory titer more than or equal to 1 Bethesda unit (BU) per milliliter, a prolonged activated partial thromboplastin time, and normal levels of other factors of the intrinsic pathway and of von Willebrand factor.

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An Inside *Blood* analysis of this article appears at the front of this issue.

The online version of this article contains a data supplement.

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Determination of FVIII-inhibitory activity

FVIII-inhibitory activity was measured in plasma using the modified Bethesda assay.[14] Plasma was heated 1 hour at 56°C. Heated plasma was incubated with an equal volume of pooled citrated human plasma (Dade-Behring) for 2 hours at 37°C. Residual FVIII activity was measured in a 1-stage clotting assay, as described. The detection limit of the assay was 0.3 BU/mL. Data were expressed in Bethesda units per milliliter, where 1 BU/mL corresponds to the inverse of the dilution of plasma that yields 50% residual FVIII activity.

Purification of IgG

IgG was isolated from plasma by affinity-chromatography on protein G-Sepharose (GE Healthcare). Intravenous immunoglobulin (IVIg, Sandoglobulin, CSL-Behring) was used as a source of normal IgG. To exclude potentially contaminating proteases, size-exclusion chromatography of patients' IgG and IVIg was performed on a Superose-12 column (GE Healthcare) equilibrated with 50mM Tris (pH 7.7), 8M urea, and 0.02% NaN_3, at a flow rate of 250 μL/min. IgG-containing fractions were pooled and dialyzed against phosphate-buffered saline-0.01% NaN_3 for 48 hours at 4°C, followed by dialysis against 50mM Tris (pH 7.7), 100mM glycine, 0.02% NaN_3, and 5mM $CaCl_2$ (catalytic buffer) for 24 hours at 4°C. We have previously demonstrated that urea-treated purified IgG retain the inhibitory activity toward FVIII.[15] The purity of IgG preparations was assessed by Western blotting under nonreducing conditions and by matrix-assisted laser desorption/ionization-time-of-flight analysis of trypsin digests of the IgG preparations. IgG was quantified by optical density measurements at 280 nm.

Proteolysis of biotinylated factors

Human recombinant FIX (BeneFIX) and FVIII (Kogenate FS or Helixate) were dialyzed against 100mM borate (pH 7.0), 150mM NaCl, and 5mM $CaCl_2$ (borate buffer) and reacted with sulfo-NHS-LC-biotin (Thermo Scientific-Pierce Protein Research Products) for 2 hours at 4°C. Biotinylated FIX and VIII were dialyzed against catalytic buffer for 3 hours at 4°C, aliquoted, and stored at −20°C until use. Biotinylated factors (185nM) were incubated in catalytic buffer with IgG (10 μg/mL, 67nM) for 24 hours at 37°C. After incubation for 24 hours at 37°C, digestion profiles were analyzed by mixing samples mixed with Laemmli buffer without β-mercaptoethanol (1:1 vol/vol), and subjecting 20 μL of each sample to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein fragments were then transferred onto nitrocellulose membrane (Schleicher & Schuell). After overnight blocking in Tris-buffered saline-0.2% Tween 20 at 4°C, membranes were incubated with streptavidin-coupled alkaline phosphatase (KPL) diluted 1:4000 in blocking buffer, for 60 minutes at room temperature. After washing in Tris-buffered saline-0.1% Tween 20, labeled proteins were revealed using the BCIP/NBT substrate kit (Pierce Chemical).

Calculation of rates of proteolysis from immunoblots

High-resolution images were acquired by scanning the immunoblots using a SnapScan 600 scanner (Agfa). Black-and-white images were converted to negatives using the Adobe Photoshop CS2 (Version 9.0.2) software. A macro was written using the National Institutes of Health image 1.62b7 software (OD macro, D. Heudes and A. Nicoletti, CRC, Inserm, France) to calculate mean image densities. Briefly, the negative images were imported into the National Institutes of Health image 1.62b7 software using the OD macro. The negative images were converted back to the positive mode by applying an arithmetic logarithmic process. The "log process" does not affect the image pixels. For calculating the rates of FIX proteolysis, we measured, for each sample, the mean density of the total area of the lane of the FIX migration profile and of the area of the protein bands with molecular weights less than 50 kDa. The percentage of FIX 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 on incubation of FIX in the presence of buffer alone was considered as the background level and was subtracted from each analysis. Data were expressed as millimoles of FIX proteolyzed per minute per mole of IgG. The lowest rate of IgG-mediated hydrolysis that could be measured was 6 μmol/min per mole of IgG. Significant differences between the rates of FIX proteolysis of patients' IgG and that of IVIg were assessed using one-way analysis of variance post-hoc test (Dunnett multiple comparison test) using Prism Version 5.0b (GraphPad Software). The reported *P* values are 2-sided. The method was essentially similar for the calculation of IgG-mediated FVIII hydrolysis.

Activation of FIX

The IgG-mediated activation of FIX was measured by its ability to activate FX. FIX (1μM) was incubated with IgG (67nM) for 23 hours at 37°C. The mixture was then incubated with FX (1μM; Haematologic Technologies), a saturating concentration (30μM) of phospholipids vesicles and $CaCl_2$ (5mM) for 1 hour at 37°C. Phospholipid vesicles (phosphatidylcholine/phosphatidylserine, 3:1) of nominal 100-nm diameter were synthesized by the membrane extrusion method.[16] Phospholipid concentrations were determined by phosphate analysis. FX activation was stopped by addition of 0.1M ethylenediaminetetraacetic acid. Activated FX formation was determined by measuring the amidolytic activity toward the synthetic substrate S2366 (0.5mM, DiaPharma Group). During the assay, less than 5% of FX was converted to activated FX, and activated FX formation was linear. Substrate conversion was monitored at 405 nm. Concentrations of generated activated FX were determined from a standard curve derived from the cleavage rate of FX by known concentrations of activated FIX (Haematologic Technologies) under the same conditions.

Kinetic parameters for IgG-mediated FIX proteolysis

FIX (800nM) was labeled with ^{125}I (PerkinElmer Life and Analytical Sciences) using iodo Gen (Pierce Chemical) as described.[17] The specific radioactivity was 0.9 μCi/μg. ^{125}I-Labeled FIX (4.5 ng) was incubated for 24 hours at 37°C with the samples (25 μg/mL IgG and 0-55.8μM of unlabeled FIX) in 40 μL of kinetic buffer (50mM Tris, 150mM NaCl, 5mM $CaCl_2$, 0.1% [wt/vol] bovine serum albumin, 0.1% [vol/vol] PEG). Samples were mixed 1:1 with Laemmli 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% SDS-PAGE in nonreducing 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, PerkinElmer Life and Analytical Sciences). Autoradiographs were scanned to allow calculation of the rate of proteolysis of labeled FIX. The data were fitted to the Michaelis-Menten equation by Prism Version 5.0b (GraphPad Software).

Analysis of N-terminal sequences

Unlabeled recombinant FIX (4 μg) was treated for 24 hours at 37°C with patients' IgG (1 μg) in 40 μL of catalytic buffer. The resultant FIX fragments were separated by 10% SDS-PAGE at 50 mA in nonreducing conditions and were transferred for 2 hours at 50 mA onto a Hybond-P PVDF membrane (GE Healthcare) in 10mM *N*-cyclohexyl-3-aminopropanesulfonic acid, 10% 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). The amount of protein sequenced ranged from 4 to 35 pmol.

Thrombin generation assay

Human plasma deficient in FVIII and devoid of platelets (PPP) (Dade-Behring, Siemens Diagnostics) was used. FVIII was supplemented to FVIII-deficient plasma at 0%, 3%, 10%, or 30% (0, 9, 30, and 90pM) of the values in normal plasma. Activated FIX was used at 0 and 0.3nM diluted in FVIII-supplemented plasma. Briefly, 80 μL of each test sample was mixed with 20 μL of N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffer or thrombin calibrator (Diagnostica Stago) on 96-well plates. Thrombin generation was triggered by adding 20 μL of fluorogenic substrate containing 102mM $CaCl_2$. Kinetics of thrombin generation were monitored for 120 minutes at 37°C using a calibrated automated thrombogram (Thrombinoscope BV) and analyzed using the appropriate software by Thrombinoscope software Version 3.0.0.29 (Synapse BV).

Table 1. Characteristics of the patients at the time of diagnosis

	No. of documented patients	No. or mean ± SD	% or range
Clinical cofactors			
Sex: male	65	40	66
Age, y	54	68.1 ± 17.0	25-92
Preexisting conditions: cancer*	55	11	20
Survival at 12 mo	52	36	69.2
Standard biologic cofactors			
FVIII activity in plasma, %	51	6.0 ± 10.9	0-59
Inhibitory titer toward FVIII, BU/mL†	65	66.9 ± 148.6	1-1050
FIX activity in plasma, %	18	117.5 ± 55.3	64-268
aPTT (measured/physiologic value)	37	2.4 ± 0.7	1.3-4.2

aPTT indicates activated partial thromboplastin time.
*Other preexisting conditions were allergic drug reactions (5), autoimmune disorders (4), dermatologic disorders (3), diabetes (1), postpartum (2), and idiopathic (14).
†Inhibitory titers were assessed using the modified Bethesda assay.

Results

Proteolytic activity of IgG against biotinylated factors VIII and IX

Plasma from 65 patients was collected at the time of diagnosis of acquired hemophilia. The mean inhibitory activity against FVIII in plasma ranged between 1 and 1050 BU/mL (Table 1). As reported,[5] IgG from some patients proteolyzed FVIII (Figure 1A, shown for patients 1, 8, and 16). IgG did not proteolyze activated FVII, prothrombin, or human serum albumin[5,18] for patients 1, 2, 8, 10, 16, 17, 20, and 32. In contrast, we observed that IgG from some patients proteolyzed FIX, yielding a major protein band migrating at 45 kDa under denaturing conditions (Figure 1B) and 2 bands migrating at 20 and 25 kDa under nondenaturing conditions (supplemental Figure 1B-C, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). IVIg (Figure 1B) and IgG purified from the plasma of 7 patients with severe hemophilia B who have developed inhibitory anti-FIX antibodies after therapeutic administration of FIX[19] did not proteolyze FIX (supplemental Figure 2). $F(ab')_2$ fragments prepared from patients' IgG also cleaved FIX, showing that the proteolytic activity lies within the variable regions of the antibodies (supplemental Figure 3). The migration profiles of FIX incubated with patients' IgG or with IVIg were subjected to densitometric analysis

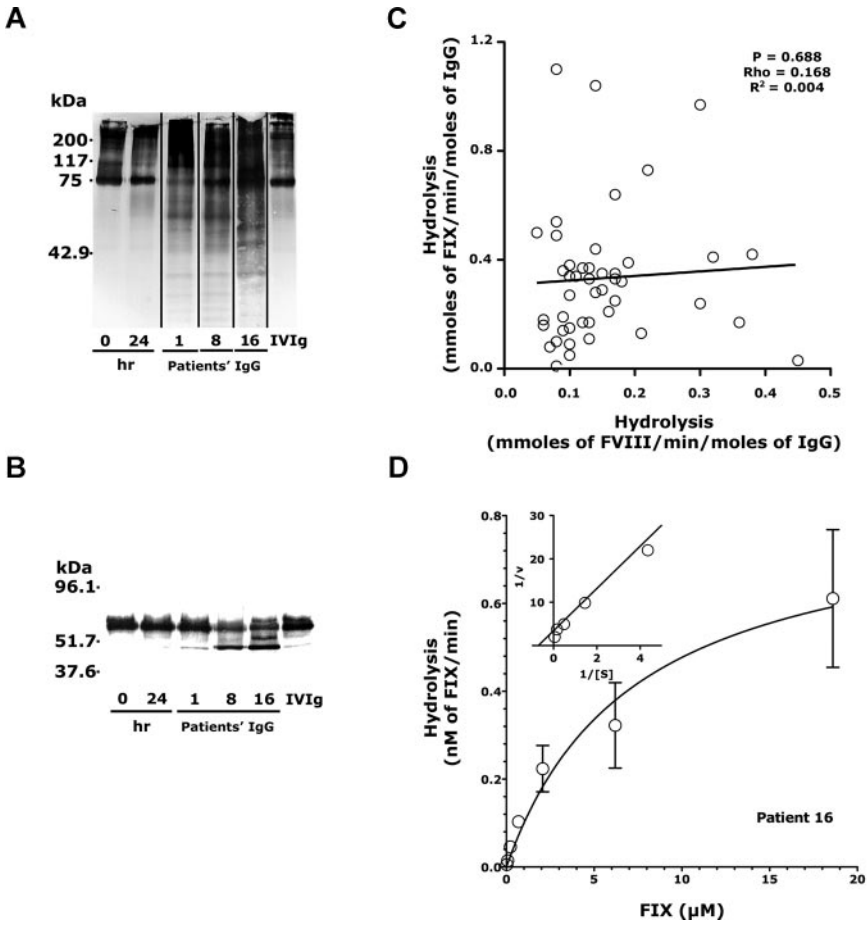

Figure 1. Proteolytic activity of IgG purified from the plasma of patients with acquired hemophilia. (A-B) IgG-mediated proteolysis of FVIII and FIX. Biotinylated human recombinant FVIII (A, 185nM) or FIX (B, 185nM) was incubated alone for 0 or 24 hours, or in the presence of IgG (67nM) for 24 hours at 37°C. IVIg was used as a source of normal IgG and as a control. Samples were subjected to 10% SDS-PAGE and transferred onto a nitrocellulose membrane, before revelation of biotinylated fragments. Vertical lines between lanes indicate a repositioned gel lane (A). (C) IgG-mediated proteolysis of FVIII versus proteolysis of FIX. The graph shows the rates of IgG-mediated proteolysis of FVIII plotted as a function of the rates of IgG-mediated proteolysis of FIX (values in Table 2). The correlation between the 2 parameters was not significant as computed using the Spearman rank correlation test. (D) Proteolysis of ^{125}I-labeled FIX by IgG in the presence of increasing amounts of unlabeled FIX. ^{125}I-Labeled FIX (4.55 ng) was incubated for 24 hours with IgG (25 μg/mL) from patient 16 in the presence of increasing concentrations of unlabeled FIX (0-20μM). Proteolysis of FIX was analyzed on a 7.5% SDS-PAGE, and autoradiographs were scanned. Rates of proteolysis of labeled FIX were calculated by densitometric analysis of the 45-kDa protein band that corresponds to activated FIX. Data are the mean of 3 independent experiments (mean ± SEM); ○ indicates empirical data. Curve indicates data fitted to the Michaelis-Menten equation ($R = 0.88$). (Insets) Reciprocal of the substrate concentration versus that of the velocity ($R = 0.99$) for the 5 highest concentrations of unlabeled FIX.

to compute the specific rates of proteolysis (Figure 1C, y-axis, Table 2). IVIg exhibited a marginal proteolytic activity of 0.06 ± 0.06 mmol/min per mole toward FIX (mean ± SD). Purified IgG from 25 of the 65 patients exhibited FIX-proteolyzing activity that was significantly higher than that of IVIg (Table 2, $P < .05$).

No FIX-binding IgG was detected in a FIX-specific enzyme-linked immunosorbent assay when whole plasma was used. In contrast, IgG purified from the plasma of 21 of 65 patients demonstrated binding activity with FIX, which was greater than that of the mean plus 1 SD measured for IVIg (supplemental Figure 4). The scored anti-FIX IgG titers did not correlate with the rates of IgG-mediated FIX proteolysis (supplemental Figure 5, $P = .384$). Rates of IgG-mediated FVIII proteolysis did not correlate with rates of IgG-mediated FIX proteolysis either (Figure 1C, $P = .688$): purified IgG proteolyzed either FVIII or FIX, both molecules, or none.

We then measured the proteolysis rate of ^{125}I-labeled FIX by IgG (167nM) from patients 16, 23, and 32 in the presence of increasing concentrations of unlabeled FIX. 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, Figure 1D inset, R = 0.88), indicating that the reaction conformed to Michaelis-Menten kinetics. The calculated average K_m and apparent V_{max} for the reactions ranged between 0.94 ± 0.25 and 7.26 ± 3.99μM, and 0.30 ± 0.08 and 0.82 ± 0.19nM/min, respectively (Table 3).

Activation of FIX by patients' IgG

We performed an electrophoretic separation under nonreduced conditions of unlabeled FIX (Figure 2A, lane 1), IgG from patients 16 and 32 (lanes 2 and 3), activated FIX (lane 4), and FIX preincubated in the presence of IgG from patients 16 and 32 (lanes 5 and 6) for 24 hours. Incubation of unlabeled FIX with patients' IgG generated a protein band with a molecular weight identical to that of activated FIX (45 kDa). N-terminal amino-acid sequencing of the 45 kDa-protein band generated on proteolysis of FIX by the IgG from patients 10, 14, 16, 23, and 32 revealed the presence of 2 protein sequences, as is expected because the heavy and light chains of FIX are linked by a disulfide bridge and comigrate as a single protein band under nonreducing conditions (supplemental Figure 1A): YNSG, which represents the N-terminal end of FIX, and VVGG, which corresponds to the N-terminal part of the catalytic domain (heavy chain) of FIX, a cleavage site for FIX-activating enzymes. The FIX fragments generated on cleavage by activated FXI are shown in supplemental Figure 1A.

To investigate whether IgG mediate activation of FIX, we developed a functional assay wherein the FIX proteolyzed by patients' IgG, was incubated in the presence of FX. The activation of FX by the generated activated FIX was monitored using a specific substrate. Importantly, the assay was independent of the presence of FVIII, the cofactor of activated FIX, so as to prevent interference by FVIII-inhibitory IgG. Incubation of FIX alone, in the presence of IVIg or IgG from an inhibitor-positive patient with hemophilia B, yielded marginal levels of activated FIX (Figure 2B, < 0.02 mmol/min per mole). In contrast, IgG from 25 of the 65 acquired hemophilia patients demonstrated a statistically significant activation of FIX compared with IVIg (Table 2, $P < .05$), with values ranging from 0.02 to 3.49 mmol/min per mole. The specific rates of IgG-mediated activation of FIX demonstrated a positive and significant correlation with the rates of IgG-mediated proteolysis of FIX (Figure 2C, $P < .001$).

Relevance of IgG-mediated activation of FIX

We then investigated the potency of activated FIX to restore thrombin generation in the context of reduced FVIII levels. Patients' IgG were estimated to generate 0.3nM of activated FIX in 24 hours, based on the calculated average kinetic parameters (Table 3). Besides, because of the presence of FVIII-inhibiting antibodies, the patients included in our study had residual levels of circulating FVIII composed between less than 1% and 30% of the value found in normal plasma (Table 1). In the absence of activated FIX (ie, using plasma that contained 90nM nonactivated FIX), we did not detect thrombin generation, even in the presence of 30% residual FVIII (supplemental Figure 6). Conversely, the addition of 0.3nM activated FIX to plasma restored thrombin generation provided the presence of more than or equal to 3% residual FVIII (Figure 2D): the time to peak was 42.5, 27.8, and 22.6 minutes when FVIII was supplemented at 3%, 10%, and 30% to FVIII-deficient plasma, respectively; it was infinite in the absence of FVIII.

In our cohort, the cumulative mortality one year after diagnosis of acquired hemophilia was 30.7% (95% confidence interval, −3.0%-64.3%). Patients who were alive one year after diagnosis had rates of IgG-mediated FIX activation that tended to be significantly higher (0.6 ± 0.9 mmol/min per mole) than those of patients who passed away during that period (0.3 ± 0.3 mmol/min per mole, $P = .067$, Figure 2E), although differences did not reach significance because of large intragroup variance.

Discussion

Acquired hemophilia is characterized by the presence of inhibitory anti-FVIII antibodies, reduced levels of circulating FVIII, and altered activated partial thromboplastin time.[20] Our finding of FIX-specific IgG in patients with acquired hemophilia is reminiscent of older work that reported acquired FIX deficiency alone or in combination with an acquired FVIII deficiency.[6-12] The presence of FIX-specific IgG in our study was, however, not associated with altered levels of circulating FIX. In agreement, no inhibition of FIX activity by the purified IgG could be detected in a functional assay (data not shown). Interestingly, detection of anti-FIX IgG by enzyme-linked immunoassay required IgG to be purified from plasma, suggesting low circulating levels of FIX-specific IgG.

Anti-FIX IgG may modulate the activity of FIX in different manners. Inhibitory anti-FIX IgG that may develop in patients with hemophilia B after the administration of therapeutic FIX to control bleedings, neutralize FIX by steric hindrance.[19,21] Although never reported in patients, some monoclonal anti-FIX IgG enhance the catalytic efficiency of activated FIX by increasing its affinity for activated FVIII.[22] We demonstrate here that IgG from some patients with acquired hemophilia significantly proteolyze and activate FIX.

Circulating activated FIX has been reported in the blood of healthy subjects. Increased levels have recurrently been associated with thrombotic conditions.[23] In physiology, FIX is hydrolyzed and activated by activated FXI and by the tissue factor/activated FVII complex; it may be cleaved and inactivated by granulocyte and neutrophil elastase,[24,25] and by plasmin.[26] Our work identifies circulating IgG with proteolytic properties as novel molecules able to hydrolyze and activate FIX under pathologic conditions. Although the estimated enzymatic kinetics of proteolytic IgG are low compared with those of classic enzymes, this may be compensated by the long half-life and substantially higher concentration of IgG

Table 2. FVIII inhibitory titers, mortality and specific rates of hydrolysis, and activation of coagulation factors by IgG purified from the plasma of 65 patients with acquired hemophilia

Patient no.	Inhibitory titer, BU/mL*	Mortality of patients†	Hydrolysis of FVIII,‡ mmol/min per mole IgG	Hydrolysis of FIX,§ mmol/min per mole IgG	Activation of FIX,‖ mmol/min per mole IgG
1	40	D	0.45 ± 0.2¶	0.03 ± 0.01	0.29 ± 0.05
2	63	A	0.15 ± 0.0	0.29 ± 0.45	0.96 ± 0.10¶
3	128	D	0.14 ± 0.1	0.44 ± 0.38¶	0.07 ± 0.00
4	114	D	0.13 ± 0.1	0.37 ± 0.06	0.03 ± 0.02
5	380	ND	0.17 ± 0.0	0.35 ± 0.19	0.03 ± 0.01
6	32	A	0.12 ± 0.1	0.37 ± 0.18	0.23 ± 0.02
7	49.4	ND	0.08 ± 0.0	0.49 ± 0.29¶	0.02 ± 0.01
8	3.1	D	0.38 ± 0.2¶	0.42 ± 0.14¶	0.95 ± 0.32¶
9	42	A	0.13 ± 0.0	0.33 ± 0.26	1.33 ± 0.34¶
10	6	A	0.17 ± 0.0	0.64 ± 0.42¶	2.01 ± 0.56¶
11	14	D	0.08 ± 0.0	0.01 ± 0.01	0.33 ± 0.12
12	2	A	0.12 ± 0.1	0.17 ± 0.13	0.70 ± 0.15¶
13	40	A	0.16 ± 0.1	0.21 ± 0.02	0.21 ± 0.05
14	2	A	0.22 ± 0.0¶	0.73 ± 0.02¶	3.43 ± 0.48¶
15	80	A	0.18 ± 0.0¶	0.32 ± 0.02	0.28 ± 0.06
16	52	A	0.14 ± 0.0¶	1.04 ± 0.14¶	3.49 ± 0.59¶
17	100	A	0.36 ± 0.1¶	0.17 ± 0.08	0.42 ± 0.04
18	10	A	0.09 ± 0.0	0.36 ± 0.27	0.13 ± 0.03
19	1.4	D	0.10 ± 0.0	0.38 ± 0.29¶	0.05 ± 0.02
20	18	D	0.08 ± 0.0	0.54 ± 0.61¶	0.19 ± 0.06
21	1050	ND	0.15 ± 0.1¶	0.35 ± 0.16	0.05 ± 0.03
22	330	D	0.14 ± 0.1¶	0.28 ± 0.05	0.59 ± 0.07¶
23	18	A	0.30 ± 0.1¶	0.97 ± 0.13¶	1.78 ± 0.17¶
24	1.3	D	0.05 ± 0.0	0.50 ± 0.17¶	0.59 ± 0.00¶
25	60	D	0.06 ± 0.0	0.16 ± 0.05	0.04 ± 0.00
26	1	A	0.06 ± 0.0	0.18 ± 0.07	0.20 ± 0.04
27	4	A	0.10 ± 0.1	0.27 ± 0.09	0.42 ± 0.04
28	56	ND	0.07 ± 0.0	0.08 ± 0.05	1.98 ± 0.17¶
29	4.5	A	0.13 ± 0.0¶	0.17 ± 0.07	0.14 ± 0.04
30	1.5	A	0.08 ± 0.0	1.10 ± 0.04¶	0.89 ± 0.28¶
31	3	A	0.11 ± 0.1	0.34 ± 0.08¶	0.07 ± 0.03
32	7	A	0.19 ± 0.0¶	0.39 ± 0.23¶	2.13 ± 0.71¶
33	362.7	A	0.08 ± 0.0	0.10 ± 0.10	0.04 ± 0.01
34	249.2	ND	0.09 ± 0.1	0.19 ± 0.18	0.06 ± 0.03
35	62	A	0.32 ± 0.0¶	0.41 ± 0.22¶	0.31 ± 0.03¶
36	54	A	0.21 ± 0.1¶	0.13 ± 0.11	0.02 ± 0.01
37	1	ND	0.13 ± 0.1	0.11 ± 0.07	0.07 ± 0.03
38	105.4	ND	0.10 ± 0.0	0.05 ± 0.04	0.04 ± 0.01
39	92.5	ND	0.09 ± 0.0	0.14 ± 0.11	0.03 ± 0.01
40	26.6	D	0.10 ± 0.1	0.09 ± 0.08	0.03 ± 0.00
41	10	D	0.17 ± 0.0	0.33 ± 0.02¶	0.85 ± 0.13¶
42	29.9	ND	0.10 ± 0.0	0.15 ± 0.12	0.03 ± 0.01
43	4.9	A	0.30 ± 0.2¶	0.24 ± 0.16	0.12 ± 0.03
44	13	A	0.10 ± 0.0	0.34 ± 0.17¶	0.26 ± 0.09¶
45	58.2	ND	0.17 ± 0.0	0.25 ± 0.14	0.03 ± 0.01
46	25	A	—	0.08 ± 0.03	0.08 ± 0.03
47	35	D	—	0.62 ± 0.08¶	0.38 ± 0.03¶
48	190	D	—	0.07 ± 0.01	0.07 ± 0.00
49	6.2	A	—	0.02 ± 0.01	0.05 ± 0.02
50	50	A	—	0.32 ± 0.12¶	0.22 ± 0.02¶
51	5.5	A	—	0.14 ± 0.06	0.09 ± 0.02
52	13.6	A	—	0.07 ± 0.02	0.10 ± 0.05
53	8	A	—	0.17 ± 0.03¶	0.16 ± 0.06¶

— indicates not documented.

*Inhibitory titers were measured in plasma using the modified Bethesda assay.

†Mortality was documented in the case of 52 of the 65 patients over a period of 365 days. A indicates alive; D, deceased; and ND, not documented.

‡The results are mean ± SD of 4 independent experiments. Rates of FVIII hydrolysis were calculated by densitometric analysis of Western blots. Spontaneous hydrolysis that occurred on incubation of FVIII in the presence of buffer alone was subtracted from each analysis. The mean coefficient of variation was 0.53 ± 0.22 (range, 0.09-1.15).

§The results are mean ± SD of 3 or 4 independent experiments. Rates of FIX hydrolysis were calculated by densitometric analysis of Western blots. Spontaneous hydrolysis that occurred on incubation of FIX in the presence of buffer alone was subtracted from each analysis. The mean coefficient of variation was 0.47 ± 0.32 (range, 0.01-1.58). The detection limit in our assay was 0.6 nM of hydrolyzed protein, equivalent to a rate of hydrolysis of 6 μmol/min per mole IgG.

‖The results are mean ± SD of 3 independent experiments. Rates of FIX activation were calculated in a functional assay as described ("Activation of FIX"). The mean coefficient of variation was 0.26 ± 0.15 (range, 0.00-0.55).

¶$P < .05$ for the comparison with intravenous immunoglobulins, using 1-way analysis of variance post-hoc test (2-tailed test).

Table 2. FVIII inhibitory titers, mortality and specific rates of hydrolysis, and activation of coagulation factors by IgG purified from the plasma of 65 patients with acquired hemophilia (continued)

Patient no.	Inhibitory titer, BU/mL *	Mortality of patients†	Hydrolysis of FVIII,‡ mmol/min per mole IgG	Hydrolysis of FIX,§ mmol/min per mole IgG	Activation of FIX,‖ mmol/min per mole IgG
54	3	A	—	0.10 ± 0.02	0.12 ± 0.03¶
55	1.5	ND	—	0.02 ± 0.01	0.06 ± 0.02
56	28	ND	—	0.04 ± 0.03	0.08 ± 0.02
57	27	A	—	0.19 ± 0.07¶	0.04 ± 0.00
58	1.7	D	—	0.12 ± 0.05	0.11 ± 0.04
59	4.4	D	—	0.10 ± 0.02	0.07 ± 0.03
60	98	A	—	0.04 ± 0.01	0.05 ± 0.02
61	45	A	—	0.29 ± 0.06¶	0.29 ± 0.05¶
62	2	A	—	0.28 ± 0.05¶	0.20 ± 0.04¶
63	1	ND	—	0.25 ± 0.02¶	0.26 ± 0.07¶
64	11.5	A	—	0.39 ± 0.01¶	0.38 ± 0.09¶
65	9	A	—	0.17 ± 0.02¶	0.16 ± 0.06¶
IVIg	0	—	0.06 ± 0.03	0.06 ± 0.06	0.01 ± 0.00

— indicates not documented.

*Inhibitory titers were measured in plasma using the modified Bethesda assay.

†Mortality was documented in the case of 52 of the 65 patients over a period of 365 days. A indicates alive; D, deceased; and ND, not documented.

‡The results are mean ± SD of 4 independent experiments. Rates of FVIII hydrolysis were calculated by densitometric analysis of Western blots. Spontaneous hydrolysis that occurred on incubation of FVIII in the presence of buffer alone was subtracted from each analysis. The mean coefficient of variation was 0.53 ± 0.22 (range, 0.09-1.15).

§The results are mean ± SD of 3 or 4 independent experiments. Rates of FIX hydrolysis were calculated by densitometric analysis of Western blots. Spontaneous hydrolysis that occurred on incubation of FIX in the presence of buffer alone was subtracted from each analysis. The mean coefficient of variation was 0.47 ± 0.32 (range, 0.01-1.58). The detection limit in our assay was 0.6 nM of hydrolyzed protein, equivalent to a rate of hydrolysis of 6 μmol/min per mole IgG.

‖The results are mean ± SD of 3 independent experiments. Rates of FIX activation were calculated in a functional assay as described ("Activation of FIX"). The mean coefficient of variation was 0.26 ± 0.15 (range, 0.00-0.55).

¶$P < .05$ for the comparison with intravenous immunoglobulins, using 1-way analysis of variance post-hoc test (2-tailed test).

in the circulation. Of note, under our experimental conditions, IgG activated up to 0.3nM of FIX in 24 hours, a value in the same range as the estimated levels of circulating activated FIX in healthy donors (ie, 0.1-0.5nM).[27] Further, given that concentrations of IgG and FIX in plasma are 10 mg/mL (67μM) and 90nM, respectively, the molar ratio of IgG antibody against FIX in plasma is 740. These molar ratios in our experiments were 0.07 to 0.36. Thus, hydrolysis was observed when these molar ratios in the experimental samples were 0.01% to 0.05% of those in normal plasma, suggesting that IgG-mediated hydrolysis is a mechanism of FIX activation by the antibodies of the patients in vivo. Activation of FIX requires proteolytic cleavage at 2 different sites. It remains to be determined whether IgG-mediated FIX activation results from a synergy between different IgG molecules each cleaving at a single location, or whether single proteolytic IgG molecules hydrolyze the 2 different sites and hence harbor promiscuous cleavage specificity.

In addition to our previous observations,[5,28,29] several pieces of evidence in the present study make it unlikely that the proteolysis of coagulation factors was caused by contaminating proteases: IgG purified from the plasma of different patients demonstrated different proteolyzing behaviors (Figure 1B); notably, IgG purified from hemophilia B patients with FIX inhibitors failed to proteolyze and activate FIX; the different purified IgG preparations selectively proteolyzed FVIII and/or FIX (Figure 1C), although they failed to proteolyze albumin, prothrombin, activated FVII,[5] or the synthetic peptide substrate for activated FX, and did not activate FX; $F(ab')_2$ fragments of patients' IgG hydrolyzed FIX, similar to the whole IgG counterparts (supplemental Figure 3). Lastly, matrix-assisted laser desorption/ionization-time-of-flight analysis of purified IgG did not reveal traces of activated FXI or other adventitious protease.

Table 3. Kinetic parameters of the hydrolysis of FIX by IgG of patients with acquired hemophilia

No. of patients	V_{max}, nM/min	K_m, μM
16	0.82 ± 0.19	7.26 ± 3.99
23	0.41 ± 0.03	0.94 ± 0.25
32	0.30 ± 0.03	1.81 ± 0.61
Mean	0.51 ± 0.08	3.34 ± 1.62

FIX was incubated at increasing concentrations (0-20μM) with IgG (167nM) of 3 patients with acquired hemophilia for 24 hours at 37°C. Hydrolysis rates were computed, and the data were fitted to the Michaelis-Menten equation to derive the apparent V_{max} and average K_m. Data are the mean ± SE of 3 independent experiments.

The physiopathologic relevance of proteolytic antibodies remains a debated issue. Antibody-mediated proteolysis has been associated with pathogenesis in asthma,[30] multiple sclerosis,[31-33] autoimmune thyroiditis,[34] and hemophilia A.[15,28] Conversely, elevated levels of IgG with amidolytic activity have been associated with positive outcome in severe sepsis,[35] or with delayed chronic allograft nephropathy in renal transplantation.[36] Here, we observe that IgG from 38.5% of the patients significantly activate FIX compared with IVIg. In our cohort, the cumulative mortality was 30.7%. Interestingly, patients who were alive 12 months after diagnosis statistically tended to have higher levels of FIX-activating IgG than patients who were deceased ($P < .1$), although the difference did not reach significance. Taken together, the data suggest that, in certain underlying pathologies, IgG-mediated FIX activation may be beneficial and represents an antihemorrhagic mechanism that compensates, at least in part, for the inhibition of endogenous FVIII by the patients' anti-FVIII autoantibodies. In support, in vitro addition of picomolar levels of activated FIX to plasma partly restores thrombin generation, provided that residual FVIII in plasma was more than or equal to 3%. Because the IgG-mediated FIX activation is reflected in marginally improved thrombin generation, it may be appropriate to analyze the effect of thrombin generation on patients' mortality in the future.

Hemorrhages in patients with acquired hemophilia are treated with FVIII, activated FVII, or activated prothrombin complexes, all of which are hampered by short half-life and/or risks for thromboembolic complications. Our results raise the issue of the

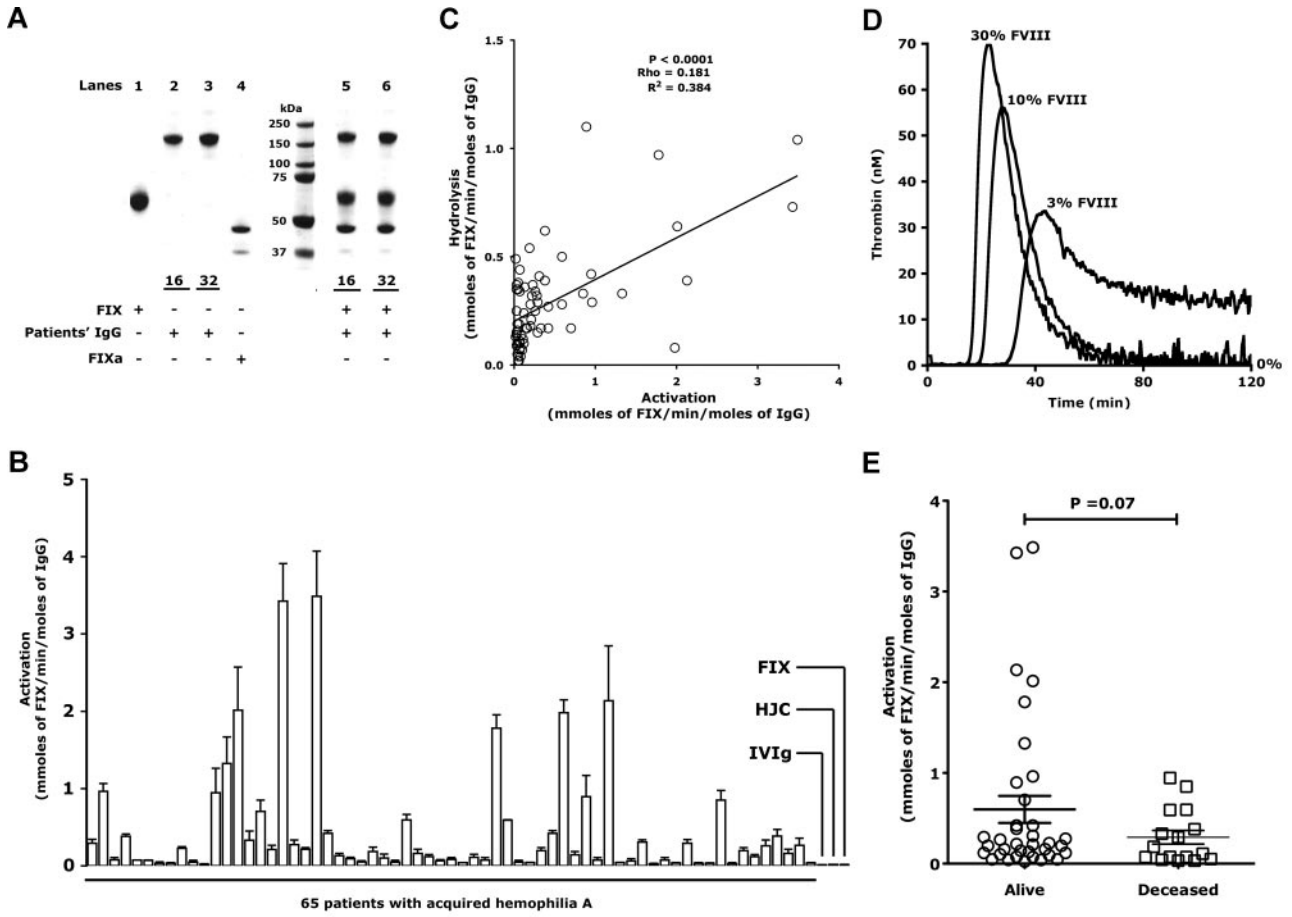

Figure 2. Activation of FIX by IgG from patients with acquired hemophilia. (A) Cleavage sites for hydrolytic IgG on FIX. Human recombinant FIX (lane 1), IgG purified from the plasma of patients 16 and 32 (lanes 2 and 3), human recombinant activated FIX (lane 4), and FIX incubated in the presence of patients' IgG (lanes 5 and 6) were subjected to 4% to 12% SDS-PAGE. Proteins were stained by colloidal Coomassie blue. (B) Activation of FIX by IgG from 65 patients with acquired hemophilia. FIX (1μM) was incubated alone (FIX) or in the presence of IVIg, IgG from a patient with congenital hemophilia B (HJC), or IgG (67nM) purified from the plasma of 65 patients with FIX-proteolyzing IgG (Table 2) for 24 hours at 37°C. Rates of formation of FIX were calculated based on the ability of the generated activated FIX to activate FX. The data are expressed as millimoles of activated FIX formed per min per mole of IgG. The data represent the means and SDs of 3 individual experiments. Purified IgG neither directly activated FX (data not shown) nor hydrolyzed the chromogenic substrate for activated FX (rates $<$ 10 fmol/min per mole of IgG). (C) IgG-mediated FIX activation correlates with FIX proteolysis. The graph shows the rates of IgG-mediated activation of FIX plotted as a function of the rates of IgG-mediated proteolysis of FIX (Table 2), both expressed in terms of millimoles of activated FIX/min per mole of IgG. The significance of correlation between the 2 parameters was computed using the Spearman rank correlation test. (D) Generation of thrombin by activated FIX in the presence of FVIII. The thrombin generation assay probes the whole intrinsic coagulation cascade from contact activation to the formation of thrombin as well as the inactivation of activated coagulation factors by plasma protease inhibitors. Tissue factor-independent thrombin generation curves were determined in human plasma that is devoid of platelets (PPP). The generation of thrombin was monitored during 120 minutes in FVIII-deficient plasma supplemented with exogenous FVIII at 0%, 3%, 10%, or 30% of the level found in normal plasma and in the presence of 0.3nM of activated FIX. The time to reach the peak of thrombin generation (referred to as time-to-peak) was computed. Results are representative of 2 independent experiments. Indicated percentage values represent adjusted FVIII levels in test plasma. (E) Relevance of IgG-mediated activation of FIX. The 52 acquired hemophilia patients with a documented survival status were divided into 2 groups based on the survival status 1 year after diagnosis. Cumulative average rates of IgG-mediated activation of FIX in deceased patients differed, although not significantly (P = .067), from those of surviving patients, as assessed using a 2-sided unpaired t test followed by Welch correction.

therapeutic relevance of the passive administration of proteolytic FIX-activating antibodies; proteolytic antibodies would advantageously combine the capacity for "turnover" that characterizes enzymatic activities, low risk for thrombotic complications because of their low catalytic rates of FIX activation, with long half-life typical of IgG molecules.

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Authorship

Contribution: B.W., O.D.C., J.D.D., H.L., J.-Y.B., J.B., S.V.K., and S.L.-D. designed the research; B.W., O.D.C., A.M., J.D.D., Y.R., V.O., and S.A. performed the research; A.B.-D., H.L., and J.-Y.B. contributed vital new reagents or analytical tools; B.W., O.D.C., A.M., A.F., T.C., S.V.K., and S.L.-D. analyzed the data; and B.W., O.D.C., J.B., and S.L.-D. wrote the paper.

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Figure S1. Factor IX activation by activated factor XI and IgG from patient 17
(A) Depiction of the activation of factor IX by activated factor XI. The figure represents the sequential cleavage between amino acid positions R145-A146 and R180-V181 of factor IX by activated factor XI. Cleavage at the first bond (R145-A146) generates a single band migrating at 56 kDa (referred to as FIXα) under non-reducing condition, whereas under reducing condition it leads to two polypeptides of sizes 20 kDa and 36 kDa. The cleavage of the second bond (R180-V181) releases the activation peptide of 11 kDa and leads to the formation of activated FIX (FIXaβ), which migrates as a 45 kDa fragment under non-reducing condition and as two polypetides of 20 kDa (light chain) and 25 kDa (heavy chain) under reducing condition. (B) Dose-dependent proteolysis of factor IX by IgG from patient 17. To identify different factor IX intermediates generated during activation by IgG, biotinylated factor IX (185 nM) was incubated with increasing concentrations of IgG (0 to 40 µg/ml) from patient 17 for 24 hr at 37°C. Samples were separated by 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose, prior to revelation of biotinylated fragments. (C) Time-dependent proteolysis of factor IX by IgG from patient 17. IgG (67 nM) from patient 17 were mixed with biotinylated factor IX (185 nM) for 0 to 48 hours at 37°C. Proteolysis of FIX was examined by SDS-PAGE and Western blot.

Figure S2. Investigation of IgG-mediated proteolysis of factor IX in patients with hemophilia B
Biotinylated human recombinant factor IX (185 nM) was incubated alone or in the presence of IgG (67 nM) purified from the plasma of 7 inhibitor-positive patients with hemophilia B, for 0 or 24 hr at 37°C. Intravenous immune globulin (IVIg) was used as a source of normal IgG and as a control (Ctl). Samples were subjected to 10 percent SDS-PAGE and transferred onto a nitrocellulose membrane, prior to revelation of biotinylated fragments.

Figure S3. Hydrolysis of FIX by $F(ab')_2$ fragments prepared from the IgG of patient 16.
$F(ab')_2$ fragments were prepared upon digestion of purified IgG with pepsin in 0.2 M Na acetate buffer (pH 4.1) for 18 hr at 37°C. The digestion was stopped on adding Tris to adjust the pH to 7.4. The preparation was dialyzed in the presence of PBS. To obtain a pure fraction of $F(ab')_2$ fragments, and to exclude potentially contaminating proteases, size-exclusion chromatography of the preparation was performed on a Superose-12 column (Amersham) equilibrated with 50 mM Tris (pH 7.7), 8 M urea and 0.02% NaN_3, at a flow rate of 250 µl/min. $F(ab')_2$-containing fractions (tested by an ELISA) were pooled and dialyzed against PBS-0.01 percent NaN_3 for 48 hr at 4°C, followed by dialysis against 50 mM Tris (pH 7.7), 100 mM glycine, 0.02 percent NaN_3, 5 mM $CaCl_2$ (catalytic buffer) for 24 hr at 4°C. Biotinylated human recombinant factor IX (185 nM) was incubated alone for 0 or 24 hr, in the presence of IgG (67 nM) or F(ab')2 preparation (67 nM) from patient 16, for 24 hr at 37°C. Intravenous immune globulin (IVIg) was used as a source of normal IgG and as a control. Samples were subjected to 10 percent SDS-PAGE and transferred onto a nitrocellulose membrane, prior to revelation of biotinylated fragments.

Figure S4. Titers of anti-factor IX IgG from patients with acquired hemophilia
An ELISA was performed using recombinant human factor IX. 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 ImmunoResearch, 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). 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 IVIg.

Figure S5. Binding of IgG to factor IX versus IgG-mediated proteolysis of factor IX
The binding of purified IgG (23 nM) from 65 patients with acquired hemophilia was tested by enzyme-linked immuno assay against human recombinant factor IX (2 µg/ml). The optical density values measured at 492 nm for IgG were plotted as a function of the calculated rates of IgG-mediated proteolysis of factor IX. The correlation between the two parameters was not significant as computed using the Spearman rank correlation test.

Figure S6. Generation of thrombin by activated factor IX in the presence of factor VIII
The thrombin generation assay probes the whole intrinsic coagulation cascade from contact activation to the formation of thrombin as well as the inactivation of activated coagulation factors by plasma protease inhibitors. Tissue factor-independent thrombin generation curves were determined in human plasma that is devoid of platelets (PPP). The generation of thrombin was monitored during 120 min in factor VIII-deficient plasma supplemented with exogenous factor VIII at 30% of the level found in normal plasma, and in the presence of 0, 0.3 and 3 nM of activated factor IX. The time to reach the peak of thrombin generation (referred to as time-to-peak) was computed. Results are representative of two independent experiments. Indicated percent values represent adjusted factor VIII levels in test plasma.

Figure S1

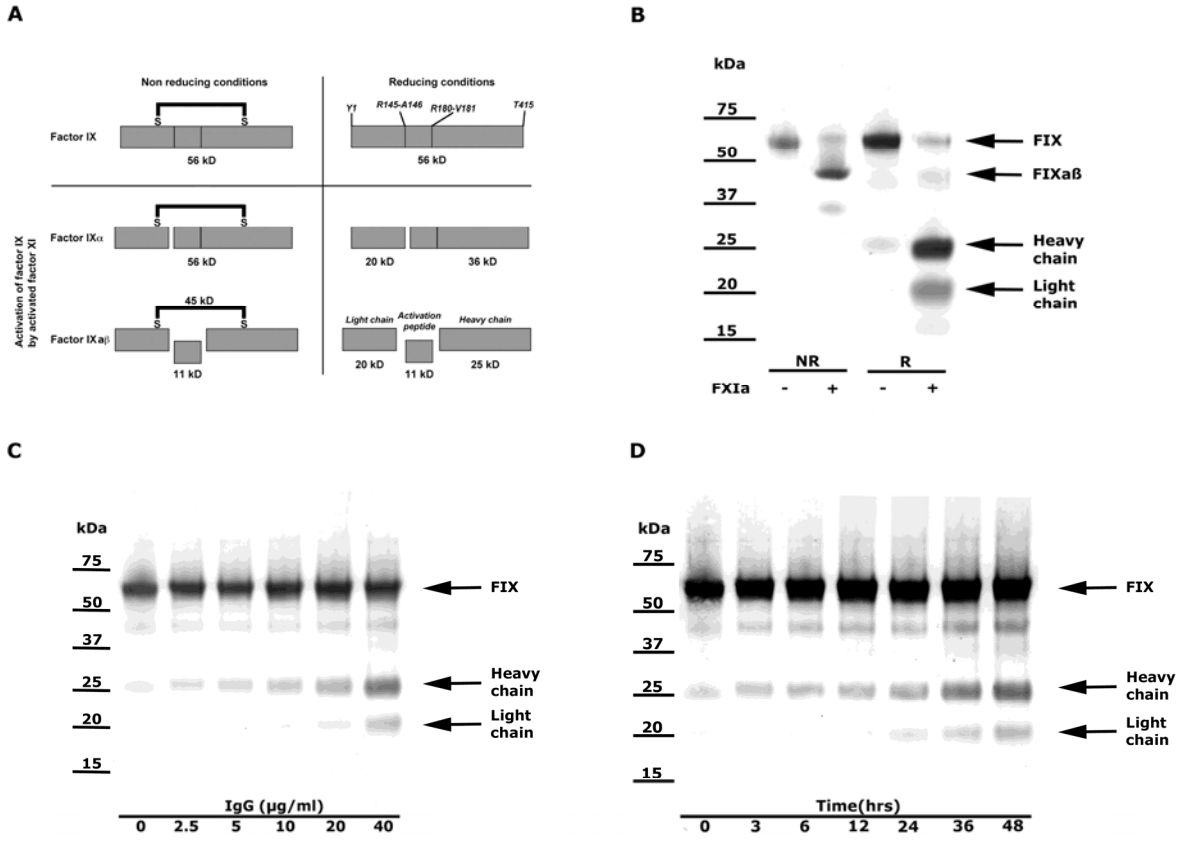

A
Non reducing conditions
Reducing conditions
Y1
R145-A146
R180-V181
T415
Factor IX
56 kD
56 kD
Factor IXα
56 kD
20 kD
36 kD
45 kD
Factor IXaβ
11 kD
Light chain
Activation peptide
Heavy chain
20 kD
11 kD
25 kD
Activation of factor IX by activated factor XI
B
kDa
75
50
37
25
20
15
FIX
FIXaß
Heavy chain
Light chain
NR
R
FXIa
- + - +
C
kDa
75
50
37
25
20
15
FIX
Heavy chain
Light chain
IgG (µg/ml)
0 2.5 5 10 20 40
D
kDa
75
50
37
25
20
15
FIX
Heavy chain
Light chain
Time(hrs)
0 3 6 12 24 36 48

Figure S2

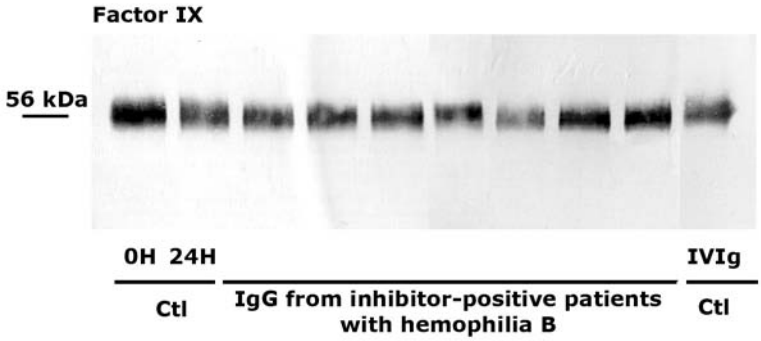

Factor IX
56 kDa
0H 24H
IVIg
Ctl
IgG from inhibitor-positive patients with hemophilia B
Ctl

Figure S3

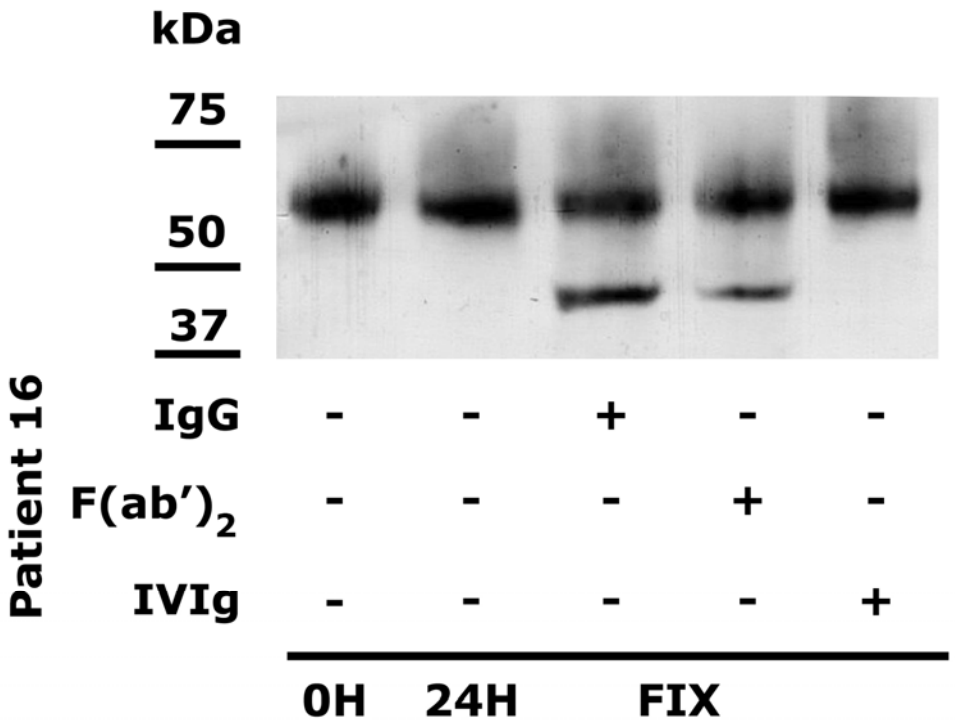


Figure S4

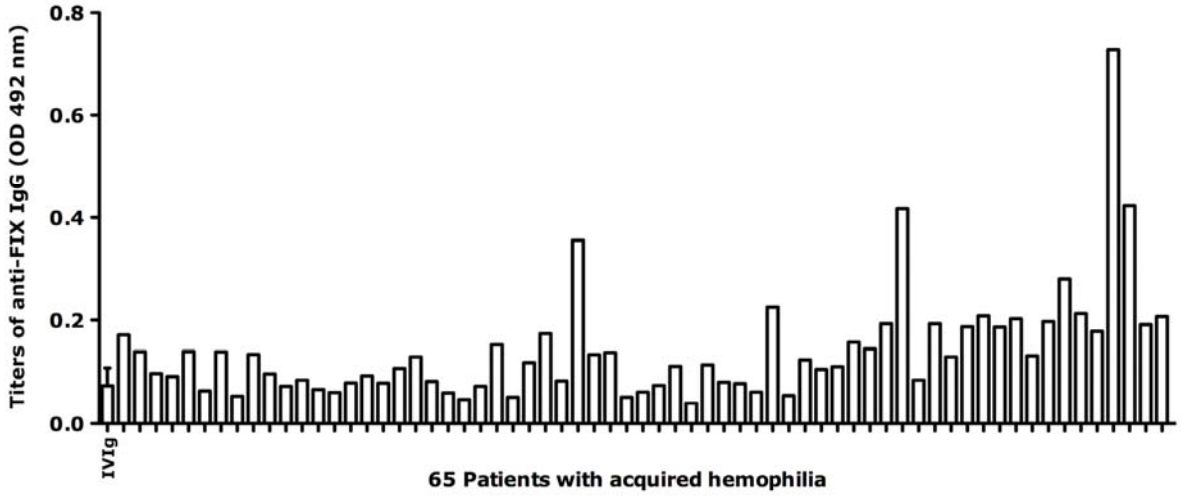

Titers of anti-FIX IgG (OD 492 nm)
0.8
0.6
0.4
0.2
0.0
IVIg
65 Patients with acquired hemophilia

Figure S5

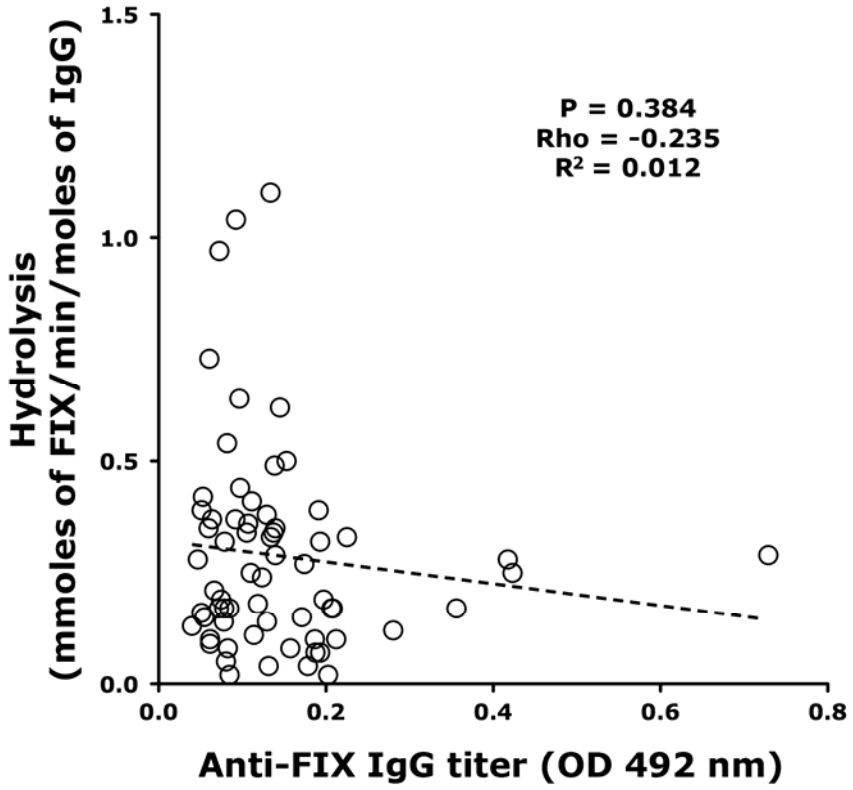
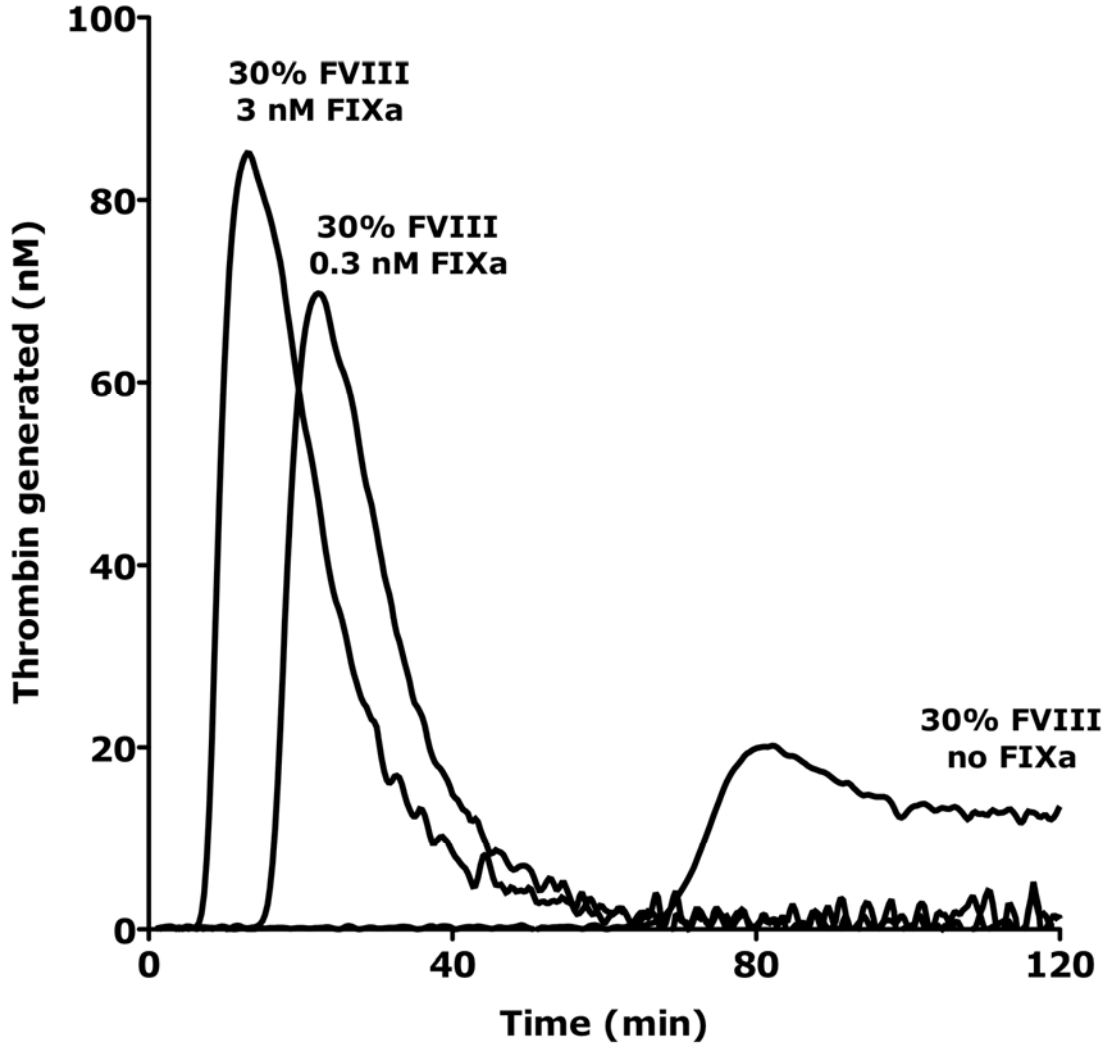

P = 0.384
Rho = -0.235
R² = 0.012
Hydrolysis
(mmoles of FIX/min/moles of IgG)
1.5
1.0
0.5
0.0
0.0
0.2
0.4
0.6
0.8
Anti-FIX IgG titer (OD 492 nm)

Figure S6


100
80
60
40
20
0
Thrombin generated (nM)
30% FVIII
3 nM FIXa
30% FVIII
0.3 nM FIXa
30% FVIII
no FIXa
0
40
80
120
Time (min)

Article 2- Résumé en Français

Titre: Evolution des niveaux d'anticorps catalytiques chez les patients ayant subi une transplantation rénale

La transplantation rénale est le traitement de choix pour les patients en insuffisance rénale terminale. Nous avons montré que de faibles niveaux d'IgG catalytiques sont un marqueur pronostique potentiel de la néphropathie chronique d'allogreffe. L'origine et l'importance physiopathologique des anticorps catalytiques sont mal connues en partie parce que ces anticorps ont été étudiés dans des cohortes relativement petites de patients atteints de maladies rares et/ou sans suivi systématique. Ici, nous avons suivi pendant deux années l'évolution des niveaux d'anticorps catalytiques dans une large cohorte de patients ayant subi une transplantation rénale. Nos résultats montrent que, avant transplantation, les patients en insuffisance rénale terminale ont des niveaux hétérogènes d'IgG hydrolysant le PFR-MCA, un substrat générique. L'hydrolyse du PFR-MCA est plus importante dans le cas des IgG des patients que dans celui d'un mélange d'IgG de donneurs sains. La transplantation rénale est marquée par une baisse drastique du niveau des IgG catalytiques en 3 mois, suivie par une augmentation progressive les 21 mois suivants. Les patients ayant de forts niveaux d'IgG catalytiques avant transplantation récupéraient des niveaux élevés d'IgG catalytiques au bout de deux ans. Par ailleurs, l'hydrolyse par les IgG d'un substrat protéique modèle, le facteur VIII de la coagulation, n'était pas corrélée à celle du PFR-MCA avant transplantation, mais l'était 24 mois plus tard. Dans leur ensemble, nos résultats suggèrent que le niveau d'IgG catalytiques circulantes en conditions pathologiques est une propriété intrinsèque du système immunitaire de chacun, et que la récupération des IgG catalytiques après transplantation est accompagnée par des modifications du répertoire des antigènes cibles.

Evolution of catalytic antibodies in patients with renal transplant

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Abstract

Renal transplant is the treatment of choice for patients with end-stage renal disease. We have previously identified low levels of catalytic IgG as a potential prognosis marker for chronic allograft rejection. The origin and physiopathological relevance of catalytic antibodies is not well understood owing to the fact that catalytic antibodies have been studied in relatively small cohorts of patients with rare diseases and/or without systematic follow-up. In the present study, we have followed the evolution of levels of catalytic IgG in a large cohort of renal transplant patients over a period of 2 years. Our results demonstrate that, prior to transplant, patients with renal failure present with heterogeneous levels of IgG hydrolyzing the generic PFR-MCA substrate. PFR-MCA hydrolysis was greater in the case of patients' IgG than in that of a preparation of pooled IgG from healthy donors. Renal transplant was marked by a drastic decrease in levels of catalytic IgG over three months followed by a steady increase during the next 21 months. Patients who displayed high levels of catalytic IgG pre-transplant recovered high levels of catalytic antibodies 2 years post-transplant. Interestingly, IgG-mediated hydrolysis of a model protein substrate, pro-coagulant factor VIII, did not correlate with that of PFR-MCA prior transplantation, while it did 24 months post-transplant. Taken together, our results suggest that the level of circulating catalytic IgG under pathological conditions is an intrinsic property of each individual's immune system, and that recovery of pre-transplant levels of catalytic IgG is accompanied by changes in the repertoire of target antigens.

Introduction

Catalytic antibodies are immunolglobulins with enzyme-like properties. The advent of hybridoma technology fuelled a remarkable progress and in the past 26 years catalytic antibodies with more than 100 tailor-made specificities have been made. From the late 80's, scientists have also investigated the pathophysiological association of catalytic antibodies in several diseased conditions. Thus, IgG with catalytic activity against vasoactive intestinal peptide, thyroglobulin, myelin basic protein, DNA/RNA and coagulation factor VIII, have been reported in pathological conditions including asthma (1), Hashimoto's thyroiditis (2), multiple sclerosis (3), systemic lupus erythmatosus (4), and hemophilia A (5). Recent findings however, suggest the presence of naturally occurring catalytic antibodies in physiology. The naturally occurring catalytic antibodies display promiscuity in antigen/substrate-specificity and are believed to act in defence mechanism against viral/bacterial pathogens. In fact, immunoglobulins of the IgA and IgM type isolated from healthy individuals are shown to possess nuclease and/or protease activity against bacterial or viral antigens (6-8). In this context, catalytic antibodies have been proposed to participate in maintaining immune homeostasis and clearing of biological wastes. Whether catalytic antibodies are a feed-back control mechanism aimed at re-establishing immune homeostasis under diseased conditions still remains elusive. In this line, our earlier investigations provide some hints. We have observed that high levels of circulating catalytic IgG correlates with a favourable outcome in some diseases. Patients with high IgG-mediated catalytic activity had a better survival rate in sepsis and a tendency towards better survival was observed in the case of patients with acquired hemophilia, that possess factor IX-activating antibodies in plasma (9, 10). Moreover, in

patients undergoing renal transplant, better graft survival correlated with the presence of high levels of IgG-mediated catalytic activity. High IgG-mediated catalytic activity as early as at 3 months was predictive of absence of chronic allograft rejection (CAN) 2 years post-transplant (11).

Despite the efforts invested to date, our understanding of the physiopathological relevance of catalytic antibodies in human health remain poor. We know that patients with different diseases generally exhibit heterogeneous levels of catalytic antibodies. We also know that the levels of catalytic antibodies may evolve with time, although not necessarily in a manner that correlates with disease progression. However, it is not clear whether high levels of catalytic antibodies in some individuals are an intrinsic property of their very immune system or are associated with peculiar disease conditions. In the present study, we followed a large cohort of patients with renal transplant for a period of 2 years, with regular and systematic blood sampling prior to transplantation as well as 3, 12 and 24 months later.

Patients and methods

Study population

From October 2008 to August 2009, we prospectively collected plasma from 100 consecutive patients 3 months following renal transplant at the Renal Transplantation Department of the Necker Hospital (Paris, France). Patients were followed-up and we also collected plasma at 12 months (92 patients) and 24 months (73 patients) post-transplant. Frozen pre-transplant plasma samples were retrieved retrospectively in the case of 59/100 patients. Clinical characteristics of the patients are depicted in Table 1. Written informed consents were obtained from each patient according to the Declaration of Helsinki.

Plasma collection

Blood was collected in citrate vacutainer tubes (BD biosciences), and centrifuged at 1500 rpm for 10 min at 20°C. Plasma was stored in aliquots at -20°C until use.

Purification of IgG

IgG were isolated from plasma by affinity-chromatography on protein G-Sepharose (Amersham Pharmacia Biotech). In brief, IgG was incubated with protein G-Sepharose overnight at 4°C, eluted using 0.2M glycine-HCl pH 2.8, dialyzed against PBS-0.02% NaN3 overnight at 4°C, and concentrated using Amicon (Millipore). A therapeutic preparation of pooled normal human IgG (intravenous Ig (IVIg); Sandoglobulin) was used as a source of control IgG. Size-exclusion chromatography of patients' IgG and IVIg was performed on a Superose-12 column (GE Healthcare Europe) equilibrated with

urea-containing buffer (50 mM Tris pH 7.7, 8 M urea and 0.02% NaN3), at a flow rate of 0.5 ml/min to exclude potentially contaminating proteases. IgG-containing fractions were then pooled and dialyzed against PBS-0.02% NaN3 for 2 days with four changes in buffer at 4°C, followed by dialysis against catalytic buffer containing 5 mM CaCl2 (pH 7.7) for 1 days with two changes in buffer at 4°C. The purity of IgG preparations was confirmed by SDS-PAGE and immunoblotting under non-reducing conditions. IgG was quantified by Bradford assay.

IgG-mediated hydrolysis of PFR-MCA

IgG (66.67 nM) were mixed with 100 µM PFR-MCA (Peptide Institute, Inc.) in 40 µl of catalytic buffer containing 5 mM CaCl2 (pH 7.7) in white 96-well U-bottom plates (Thermo Scientific) and incubated in the dark for 24 h at 37°C. Hydrolysis of the PFR-MCA substrate was determined by the fluorescence of the leaving group (aminomethylcoumarin; λem 465 nm, λex 360 nm) using a spectrofluorometer (GENios; Tecan Trading). Fluorescence values were compared with a standard curve of free MCA and the corresponding quantities of released MCA were computed. At each time point, background release of MCA, measured in wells containing the substrate alone, was subtracted from the value observed in the presence of the Abs. Data are expressed as the quantity of released MCA computed at time 0 subtracted from the quantity of released MCA computed at a given time point per amount of time per amount of IgG.

Biotinylation of FVIII

Recombinant human factor VIII (FVIII, Kogenate FS, BayerPharma, Lille, France) was

reconstituted in distilled water to a final concentration of 600 μg/ml, desalted by dialyzing against borate buffer (100 mM borate (pH 7.0), 150 mM NaCl, and 5 mM CaCl2). Sulfo-NHS-LC-biotin (440 μl at 25 μg/ml) was allowed to react with 600 μg of FVIII with gentle agitation in the dark for 2 h at 4°C. Biotinylated FVIII was dialyzed against catalytic buffer containing 5 mM CaCl2 for 3 h at 4°C, aliquoted, and stored at -20°C until use.

Hydrolysis of biotinylated FVIII

Biotinylated FVIII (185 nM) was incubated in 40 μl of catalytic buffer containing 5 mM CaCl2 with the purified patients' IgG (10 μg/ml, 66.67 nM) in the dark for 24 h at 37°C. Samples were mixed with Laemmli's buffer without 2-ME (1:1, v/v) and 25 μl of each sample was subjected to 10% SDS-PAGE. Protein fragments were then transferred onto nitrocellulose membranes (Schleicher & Schuell's Microscience). Following overnight blocking in TBS containing 0.2% Tween 20 at 4°C, membranes were incubated with streptavidin-coupled alkaline phosphatase (Southern Biotech) diluted 1:3000 in blocking buffer, for 60 min at room temperature. After washing in TBS containing 0.1% tween-20 and TBS, labeled FVIII was revealed using the BCIP/NBT kit (Kirkegaard & Perry Laboratories). Blots were scanned using a scanner (EPSON Perfection V10) and rates of hydrolysis were calculated by densitometric analysis.

Statistics

The statistical comparisons of groups of patients were performed using the non-parametric Mann-Whitney test, with two-tailed P values, unless indicated.

Results

IgG from pre-transplant patients show heterogeneous levels of catalytic activity, which is not related to particular underlying pathology

We collected plasma from 100 consecutive renal-transplant patients after 3 months of transplant and followed-up to obtain plasma at 12 (92 patients) and 24 months (73 patients) post-transplant. Pre-transplant plasma samples from 59 patients were retrieved retrospectively. Overall, 27 patients were lost during the 24 months of the study period. The cohort included as many men as women, with a mean age of 48.3±1.5 years (mean±SEM; range: 21 to 83). Causes for end-stage renal dysfunction included diabetes (4% of the patients), vasculopathy (8%), glomerulopathy (24%), uropathy (23%), interstitial nephropathy (15%) or were not known (26%). Fifteen and 2 patients had had one or two previous transplants, respectively. Fifteen and 2 patients had had one or two previous transplants, respectively.

IgG was purified and tested for hydrolysis of the peptide PFR-MCA, a surrogate substrate for catalytic antibodies with serine protease-like activity (12). The absence of contamination of the IgG samples by adventitious proteases was ensured by the use of a double-step purification procedure that involves a step of purification based on affinity and a step of purification based on protein size under denaturing conditions. Incubation of patients' IgG with PFR-MCA resulted in hydrolysis of the peptide and release of the fluorescent MCA moiety. The released fluorescence allowed for the calculation of rates of hydrolysis.

Hydrolysis of PFR-MCA was dose- and time-dependent (data not shown). Pooled IgG from healthy individuals (IVIg) demonstrated a marginal hydrolysis of PFR-MCA with

an activity of 0.65±0.03 fmol/min per pmol (mean±SEM for 29 repeats). Irrespective of the time-point considered, IgG from renal-transplanted patients demonstrated significantly higher hydrolysis rates of PFR-MCA than IVIg (Figure 1A). There was no significant difference in the levels of PFR-MCA-hydrolyzing IgG at any time point with respect to the sex, the age, the transplantation rank, the cause for end-stage renal failure and the time of dialysis prior to transplant (data not shown and Table 1).

The levels of PFR-MCA hydrolyzing IgG were extremely heterogeneous prior to transplantation, with a mean activity of 6.6±0.9 fmol/min/pmol (mean±SEM; coefficient of variation: 1.04, Figure 1A). To investigate whether high or low levels of PFR-MCA hydrolyzing IgG were associated with a particular disease condition, we compared the hydrolysis rates between patients with different causes for end-stage renal failure. IgG from patients with uropathy displayed the lowest mean rate of PFR-MCA hydrolysis, that was statistically different from that of IgG from patients with interstitial nephropathy (3.7±0.7 vs 7.9±1.2 fmol/min/pmol, P=0.003, Figure 1B). Of note, several underlying pathologies had only a low number of cases, thus hampering powerful statistical comparison.

IgG-mediated catalytic activity varies overtime in renal-transplant patients

A longitudinal follow-up of the levels of PFR-MCA-hydrolyzing IgG was performed to determine the evolution of IgG-mediated catalytic activity during the course of the disease. The number of patients at each time point (0, 3, 12 and 24 months) was 59, 100, 92 and 73, respectively. The differences in the rates of IgG-mediated catalytic activity between each group were evaluated using the two-tailed Mann Whitney test. As

compared to the rates of IgG-mediated catalytic activity pre-transplant, the rates decreased sharply 3 months post-transplant (6.6±0.9 vs 2.4±0.2 fmol/min/pmol, P<0.0001, Figure 1A). However, an increase in the rates of hydrolysis of PFR-MCA was observed at 12 months post-transplant as compared to 3 months (3.2±0.3 vs 2.4±0.2 fmol/min/pmol, P=0.008). The rates of hydrolysis further increased significantly at 24 months (5.1±0.6 fmol/min/pmol) in comparison to 3 months (P<0.0001) and 12 months (P=0.016). There was no difference in the rates of IgG-mediated catalytic activity between patients prior to transplant and 24 months later (6.6±0.9 vs 5.1±0.6 fmol/min/pmol), indicating that the pre-existing levels of PFR-MCA-hydrolyzing IgG had been recovered in the due course of time.

Linear regression analysis between the groups of patients pre-transplant and 24 months post-transplant, showed a significantly positive correlation in the rates of IgG-mediated PFR-MCA hydrolysis (P<0.001, R^2=0.23, Figure 1C). No correlation in IgG-mediated catalytic activity was observed between the groups of patients at other time points. To confirm that patients with elevated levels of catalytic antibodies before transplant recover elevated levels two years later, we divided the patients into quartiles based on the rates of IgG-mediated PFR-MCA hydrolysis measured in pre-transplant samples. At each time point, the IgG-mediated catalytic activity of the upper quartile was compared with the rates of catalytic activity of the cumulated lower three quartiles (Figure 1D). The rate of IgG-mediated catalytic activity in the upper quartile of patients was significantly high both pre-transplant (12.03±1.6 vs 2.7±0.2 fmol/min/pmol, P<0.0001) and 24 months post transplant (6.8±1.2 vs 4.6±0.7, fmol/min/pmol, P=0.009).

IgG-mediated FVIII and PFR-MCA hydrolysis correlate at 12 months but not prior to transplant

IgG from patients' plasma prior to transplant, and 3 and 12 months post-transplant were tested for their ability to hydrolyze human recombinant FVIII. Patients' IgG were incubated with biotinylated FVIII and profiles of FVIII hydrolysis were revealed by Western blotting (Fig 2A). The rates of IgG-mediated FVIII hydrolysis were calculated by densitometric analysis after subtracting the amount of FVIII hydrolysed spontaneously in the absence of IgG. Patients' IgG demonstrated heterogeneous profiles of FVIII hydrolysis (Fig 2A). The rates of hydrolysis of FVIII among pre-transplant patients were highly heterogeneous. As observed for PFR-MCA hydrolysis, the rate of IgG-mediated FVIII hydrolysis decreased significantly 3 months post-transplant (157±9.8 vs 43.8±10.2 μmol/min/mol, $P<0.0001$, Fig 2B), and recovered after 12 months (113.7±17.6 μmol/min/mol). Pooled IgG from healthy individuals demonstrated a marginal hydrolysis of FVIII with an activity of 11.1±1.2 μmol/min/mol (mean±SEM for 22 repeats).

While the mean rate of hydrolysis of PFR-MCA and FVIII by patients' IgG was relatively high prior to transplant, no correlation was observed between IgG-mediated PFR-MCA hydrolysis and IgG-mediated FVIII hydrolysis ($P=0.17$, $R^2=0.03$, Figure 2C). The longitudinal follow-up however demonstrated a correlation tendency at three months ($P=0.053$, $R^2=0.01$), which became significant 12 months post transplantation ($P<0.0001$, $R^2=0.4$, Figure 2D).

Discussion

The results are discussed in the main discussion part of the thesis.

Acknowledgements

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Authors contributions

Designed the work : AM, AF, CL, OT, SVK, SLD

Performed the research : AM, IP, CD, BW, SPL

Contributed material : CL

Analyzed the data : AM, IP, LG, SPL, OT, SLD

Wrote the paper : AM, LG, SLD

The authors of this manuscript have no conflicts of interest to disclose

Tables

Table 1. Patients' characteristics

	Nb (%) or median [range]
Age (years)	**100**
	48 [21-83]
Sex	**100**
Females	50
Males	50
Cause for end-stage renal failure	**100**
Diabetes	4
Vascular diseases	8
Glomerulopathy	24
Uropathy	23
Interstitial nephropathy	15
Unknown	26
HLA reactive antibodies	**68**
HLA1	18 (27)
ND	32
HLA2	29 (43)
ND	32
Both HLA1 and HLA2	10 (15)
ND	34
HLA mismatch	**98**

0	5
1	10
2	22
3	12
4	32
5	16
6	1
Risk group	**99**
1	15
2	40
3	44
Previous kidney transplant	**100**
None	82
1	15
2	2
ND	1

ND: not documented

Figure legends.

Figure 1. Longitudinal analysis of catalytic IgG in patients undergoing renal transplant. Panel A. Evolution of IgG-mediated catalytic activity in patients with renal transplant. IgG were purified from the plasma of patients collected prior to kidney transplant (n=59), as well as 3 (n=100), 12 (n=92) and 24 (n=73) months following transplantation. Purified IgG (66.67 nM) were incubated with the PFR-MCA substrate (100μM) at 37°C for 24 hr. PFR-MCA hydrolysis was quantified by measuring the fluorescence of the leaving fluorescent MCA moiety, and is expressed in fmol of hydrolyzed substrate as a function of time per pmol of IgG. The hydrolysis of PFR-MCA by pooled IgG from healthy donors is depicted by a dotted line. Hydrolysis was compared between groups using the two-tailed Mann Whitney U test: *: P=0.015; **: P=0.004; ***: P<0.0001). **Panel B.** Catalytic IgG in patients with different causes for end-stage renal failure. The study cohort included 2 patients with diabetes, 3 with vasculopathy, 13 with glomerulopathy, 15 with uropathy, 11 with interstitial nephropathy and 15 with unknown cause for end-stage renal failure. Statistical significances were assessed using the two-tailed Mann Whitney U test. **Panel C.** Correlation between IgG-mediated PFR-MCA hydrolysis prior to transplantation and 24 months later. The rates of IgG-mediated PFR-MCA hydrolysis measured prior to renal transplant and after 24 months were positively correlated as analyzed by linear regression. **Panel D**. Longitudinal follow-up of the patients displaying high levels of catalytic IgG. The pre-transplant patients were divided into quartiles and the mean rates of PFR-MCA hydrolysis of the upper quartile was compared with that of the pooled remaining quartiles. Hydrolysis was compared

between groups using the two-tailed Mann Whitney U test (*: P=0.03; ***: P<0.0001 or P= 0.0004).

Figure 2. Hydrolysis of FVIII by IgG from renal-transplant patients. Panel A. IgG-mediated FVIII hydrolysis. Biotinylated recombinant human FVIII (185 nM) was incubated alone (lane 1) or in the presence of IgG (66.67 nM) from 7 randomly selected renal-transplanted patients (lanes 1-7) for 24 hr at 37°C. Pooled normal IgG from healthy donors (IVIg) was used as control IgG. FVIII was subjected to 10% SDS-PAGE and transferred onto a nitrocellulose membrane before revelation of biotinylated fragments. **Panel B.** Longitudinal follow-up of FVIII-hydrolyzing IgG. Rates of IgG-mediated FVIII hydrolysis were obtained by densitometric analysis of the blots (Panel A). Results are expressed as μmol of hydrolyzed FVIII per min per mol of IgG. Statistical differences where assessed using the two-tailed Mann Whitney U test: ***: P<0.0001 or P=0.0004; **: P<0.001. **Panels C and D.** Correlation of rates of PFR-MCA hydrolysis and FVIII hydrolysis by IgG prior transplant (Panel C; ns: not significant) and 24 months later (Panel D).

Figure 1.

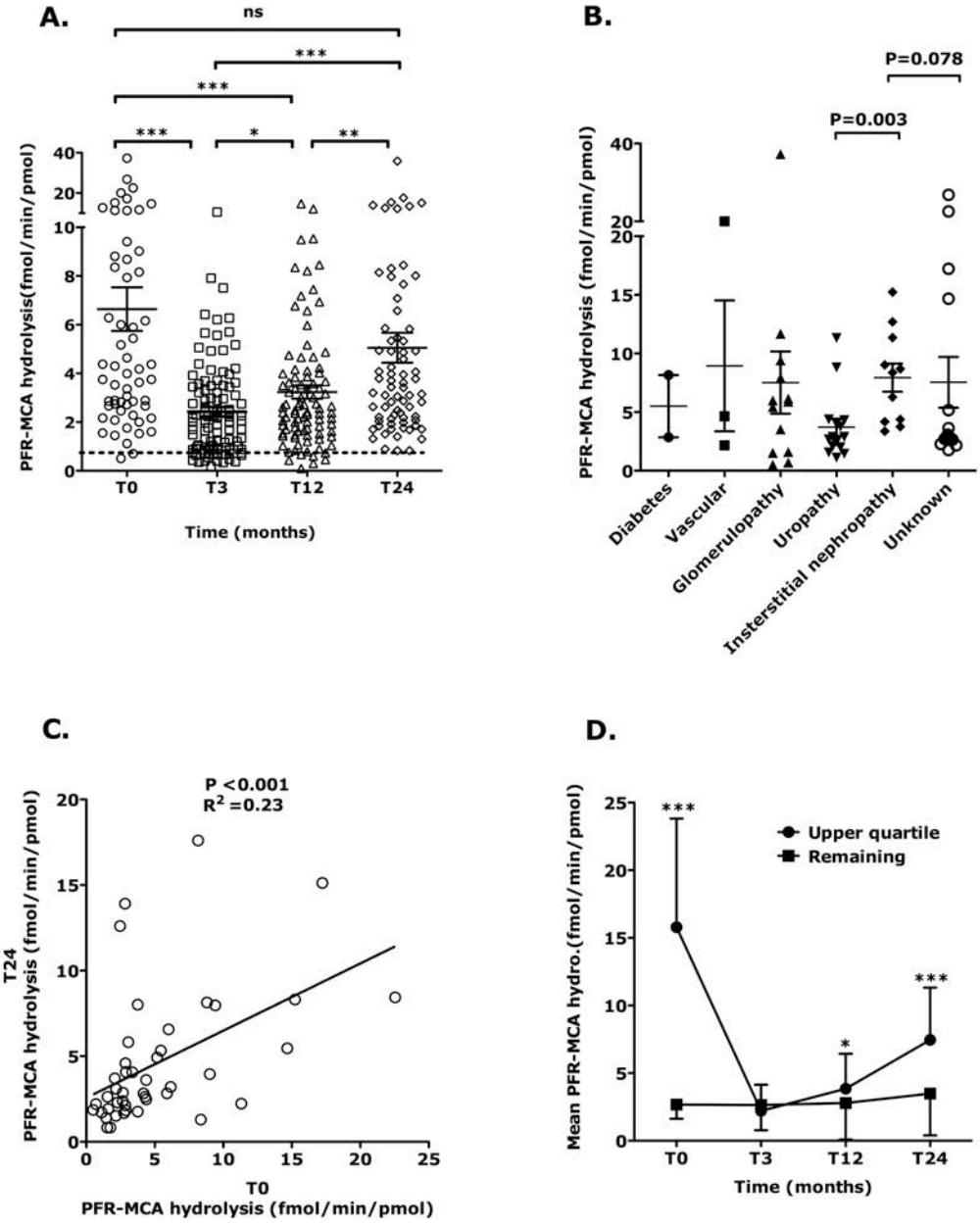
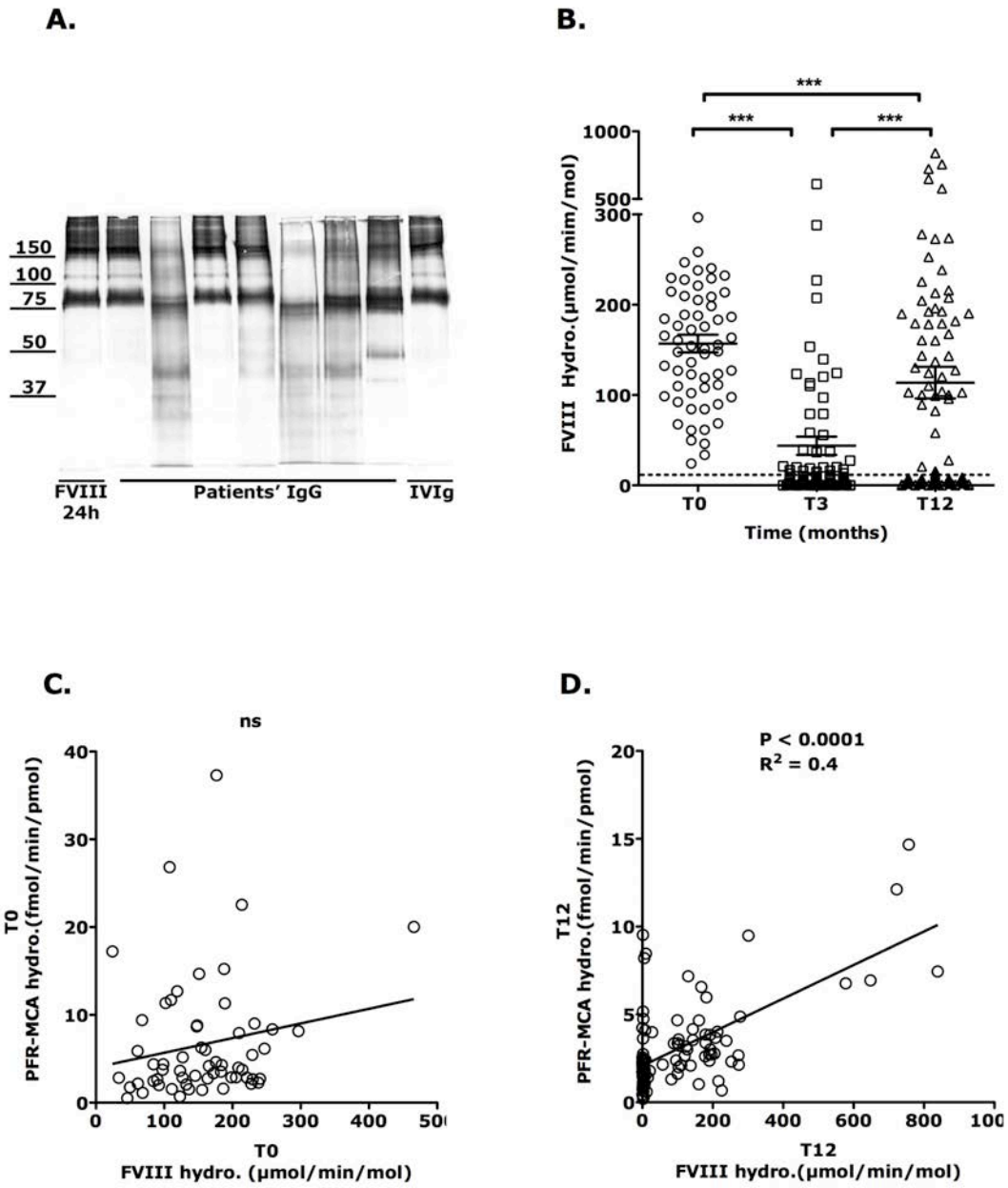

A.
ns
***
***
***
*
**
PFR-MCA hydrolysis(fmol/min/pmol)
T0
T3
T12
T24
Time (months)
B.
P=0.078
P=0.003
PFR-MCA hydrolysis (fmol/min/pmol)
Diabetes
Vascular
Glomerulopathy
Uropathy
Insterstitial nephropathy
Unknown
C.
P <0.001
R2 =0.23
T24
PFR-MCA hydrolysis (fmol/min/pmol)
T0
PFR-MCA hydrolysis (fmol/min/pmol)
D.
Upper quartile
Remaining
Mean PFR-MCA hydro.(fmol/min/pmol)
Time (months)

Figure 2.


A.
150
100
75
50
37
FVIII 24h
Patients' IgG
IVIg
B.
***
***
***
1000
500
300
200
100
0
FVIII Hydro.(µmol/mim/mol)
T0
T3
T12
Time (months)
C.
ns
40
30
20
10
0
T0 PFR-MCA hydro.(fmol/min/pmol)
0
100
200
300
400
T0
FVIII hydro. (µmol/min/mol)
D.
P < 0.0001
R2 = 0.4
20
15
10
5
0
T12 PFR-MCA hydro.(fmol/min/pmol)
0
200
400
600
800
T12
FVIII hydro.(µmol/min/mol)

Article 3- Résumé en Français

Titre: Réduction du titre des anticorps catalytiques chez les patients traités aux IgIV ayant subi une transplantation rénale

Les anticorps catalytiques sont des immunoglobulines dotées d'activité enzymatique. Les IgG catalytiques ont été décrites dans différentes pathologies inflammatoires et autoimmunes chez l'homme. En particulier, de faibles niveaux d'IgG catalytiques ont été proposés comme marqueur pronostique du rejet chronique d'allogreffe chez les patients subissant une transplantation rénale. La transplantation rénale est le traitement de choix pour les patients en insuffisance rénale terminale. Chez les patients possédant des anticorps spécifiques du donneur, les immunoglobulines intraveineuses, une préparation thérapeutique d'IgG humaines normales (IVIg), sont utilisées, seules ou avec d'autres traitements immunosuppresseurs, pour désensibiliser les patients et prévenir le rejet de greffe aigu. Dans cette étude, nous avons suivi sur une période de 24 mois les niveaux d'IgG catalytiques dans une large cohorte de patients subissant une transplantation rénale. Vingt-quatre pourcents des patients avaient reçu des IVIg au moment de la transplantation. Nos résultats démontrent une réduction marquée des niveaux d'IgG catalytiques chez tous les patients trois mois après transplantation ; la diminution étant significativement plus marquée chez les patients traités avec les IVIg. Ces travaux suggèrent que la prévention du rejet aigu à l'aide des IVIg induit une baisse transitoire des niveaux d'IgG catalytiques, qui pourrait remettre en cause l'utilisation de la mesure des niveaux d'IgG catalytiques comme marqueur pronostique de la néphropathie chronique d'allogreffe.

Reduction of catalytic antibody titers in IVIg-treated patients undergoing renal transplant

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Abstract

Catalytic antibodies are immunoglobulins endowed with enzymatic activity. Catalytic IgG has been reported in several human autoimmune and inflammatory diseases. In particular, low levels of catalytic IgG have been proposed as a prognostic marker for chronic allograft rejection in patients undergoing kidney transplant. Kidney allograft is treatment of choice for patients with end-stage renal failure. Intravenous immunoglobulins, a therapeutic pool of normal human IgG, is used in patients with donor-specific antibodies, alone or in conjunction with other immunosuppressive treatments, in order to desensitize the patients and prevent the development of acute graft rejection. In the present study, we followed for a period of 24 months the levels of catalytic IgG in a large cohort of patients undergoing kidney transplantation. Twenty-four percent of the patients received IVIg at the time of transplantation. Our results demonstrate a marked reduction in the levels of catalytic antibodies in all patients three months following kidney transplant. The decrease was significantly more pronounced in patients receiving adjunct IVIg therapy. The results suggests that prevention of acute graft rejection using intravenous immunoglobulins induces a transient reduction in the levels of catalytic IgG, thus potentially jeopardizing the use of levels of catalytic antibodies as a prognosis marker for chronic allograft nephropathy.

Introduction

Catalytic antibodies are immunoglobulins that are endowed with enzymatic activity (1). The first examples of catalytic antibodies were obtained following the active immunization of experimental animals with appropriate immunogens, referred to as transition state analogs (1-3). Since then, a series of approaches has been elaborated to generate antibodies with desired enzymatic activities (4-6). Antibodies with enzymatic properties however also develop spontaneously *in vivo*. Thus, IgG able to hydrolyze the vasoactive intestinal peptide, DNA, thyroglobulin or pro-coagulant factor VIII have been described in patients with asthma, systemic lupus erythematosus, Hashimoto's thyroiditis and hemophilia A, respectively (7-11). Because catalytic antibodies in the human had been reported under pathological conditions, it was long thought that they are endowed with a pathogenic role, or that, at least, they are a hallmark of immune dysregulation and uncontrolled inflammation (12). However, catalytic antibodies of the IgM, IgG and IgA isotypes have since been reported in normal blood, in the milk of healthy mothers and in saliva (13-15). Under physiological conditions, the antigen/substrate specificity of catalytic antibodies is unknown and the latter are generally probed using surrogate synthetic peptide substrates (16). It was proposed that catalytic antibodies may participate in immune homeostasis and in clearance of biological wastes (17, 18), in line with the hypothesis proposed by P Grabar regarding naturally occurring antibodies (19). Interestingly, we have recently demonstrated a correlation between the increased prevalence of catalytic IgG and positive outcome in several human diseases. Thus, increased levels of IgG capable of hydrolyzing the synthetic tri-peptide substrate for serine proteases - proline-phenylalanine-arginine-methyl-coumarinamide (PFR-MCA),

were found at the time of diagnosis in patients who had survived septic shock three weeks later (20). Similarly, we documented the presence of PFR-MCA-hydrolyzing IgG in the plasma of patients undergoing renal transplant (21). Low levels of catalytic IgG 3 months following transplantation were predictive of chronic allograft nephropathy (CAN) 2 years down the lane, suggesting that IgG-mediated PFR-MCA hydrolysis may be used as a prognosis marker for CAN in renal-transplanted patients (21).

Kidney allograft is treatment of choice for patients with end-stage renal failure (22, 23). Short and long-term renal graft survival is particularly compromised in patients transplanted across the HLA barrier (24). In particular, some patients, referred to as "sensitized" patients possess anti-HLA antibodies, also referred to as "donor-specific antibodies" (DSA), of either natural origin, or induced by multiple pregnancies, blood transfusion and/or previous transplant. Because sensitized patients are at a higher risk of developing humoral acute graft rejection, several strategies have been developed in the last decades to desensitize the patients. Among these, the use of intravenous immunoglobulins for therapeutic use (IVIg), either alone or in conjunction with plasmapheresis and/or monoclonal anti-CD20 antibody-mediated B-cell depletion (Rituximab), has allowed to increase the rate of transplant of highly sensitized patients, while reducing the time to transplant and the risk of acute rejection (24-26). IVIg represents a pool of normal human IgG purified from the plasma of several thousands of healthy donors. It is endowed with immuno-regulatory and anti-inflammatory properties, justifying their use in a plethora of pathological conditions (27).

Fluctuations with time of levels of catalytic antibodies in the human population and under pathological state have never been studied in a systematic manner (20, 28, 29). Likewise,

the size of the cohorts of individuals included in studies on catalytic antibodies has always been limited, mostly owing to the fact that catalytic antibodies had been described in patients with rare diseases originating from varied clinical centers (28, 29). In the present intermediate study, we followed the levels of catalytic IgG in a large cohort of patients with renal transplant for a period of 24 months post-transplant. The results document higher levels of PFR-MCA-hydrolyzing IgG in patients prior to transplant, as compared with pooled IgG from healthy individuals. A drastic reduction in the levels of catalytic antibodies is observed in all patients three months following kidney transplant. The decrease is significantly more pronounced in patients receiving adjunct IVIg therapy. Taken together, the results suggests that the treatments dedicated at preventing acute graft rejection induce a transient reduction in the levels of catalytic, thus potentially jeopardizing the use of levels of catalytic antibodies as a prognosis marker for CAN.

Patients and methods

Study population

From February 2008 to August 2009, we prospectively collected plasma from consecutive patients 3 months following renal transplant at the Renal Transplantation Department of the Necker Hospital (Paris, France). Plasma was also collected 12 months and 24 months post-transplant. Frozen pre-transplant plasma samples were retrieved retrospectively when available (n=59). Clinical characteristics of the patients were collected at the same time-points during physical examination and are depicted in Table 1. Written informed consents were obtained from each patient according to the Declaration of Helsinki. Some patients had received IVIg at the time of renal transplant. The protocol for IVIg treatment consisted in 2g per kg body weight Endobulin® (Baxter, Maurepas) over a 96-hr period of time on the day of transplant, on day 21, 42 and 63 after kidney.

Plasma collection

Blood was collected in citrate Vacutainer® tubes (BD biosciences), and centrifuged at 1500 rpm for 10 min at 20°C. Plasma was stored in aliquots at -20°C until use.

Purification of IgG

IgG were isolated from serum by affinity-chromatography on protein G-Sepharose (Amersham Pharmacia Biotech). In brief, IgG was incubated with protein G-Sepharose overnight at 4°C, eluted using 0.2M glycine-HCl pH 2.8, dialyzed against PBS-0.02% NaN3 overnight at 4°C, and concentrated using Amicon (Millipore). A therapeutic preparation of pooled normal human IgG (intravenous Ig (IVIg); Sandoglobulin®) was

used as a source of control IgG. Size-exclusion chromatography of patients' IgG and IVIg was performed on a Superose-12 column (GE Healthcare Europe) equilibrated with urea-containing buffer (50 mM Tris pH 7.7, 8 M urea and 0.02% NaN3), at a flow rate of 0.5 ml/min to exclude potentially contaminating proteases. IgG-containing fractions were then pooled and dialyzed against PBS-0.02% NaN3 for 2 days with four changes in buffer at 4°C, followed by dialysis against catalytic buffer containing 5 mM CaCl2 (pH 7.7) for 1 days with two changes in buffer at 4°C. The purity of IgG preparations was confirmed by SDS-PAGE and immunoblotting under non-reducing conditions. IgG was quantified by Bradford assay.

IgG-mediated hydrolysis of PFR-MCA

IgG (66.67 nM) were mixed with 100 μM PFR-MCA (Peptide Institute, Inc.) in 40 μl of catalytic buffer containing 5 mM CaCl2 (pH 7.7) in white 96-well U-bottom plates (Thermo Scientific) and incubated in the dark for 24 h at 37°C. Hydrolysis of the PFR-MCA substrate was determined by the fluorescence of the leaving group (aminomethylcoumarin; λem 465 nm, λex 360 nm) using a spectrofluorometer (GENios; Tecan Trading). Fluorescence values were compared with a standard curve of free MCA and the corresponding quantities of released MCA were computed. At each time point, background release of MCA, measured in wells containing the substrate alone, was subtracted from the value observed in the presence of the Abs. Data are expressed as the quantity of released MCA computed at time 0 subtracted from the quantity of released MCA computed at a given time point per amount of time per amount of IgG.

Statistics

The statistical comparisons of groups of patients treated with IVIg and of patients not treated with IVIg were performed using the non parametric Mann-Whitney test, with two-tailed p values.

Results

Circulating IgG from renal-transplanted patients hydrolyze PFR-MCA

Plasma was collected from 100 consecutive renal-transplanted patients 3, 12 and 24 months post-transplant between February 2008 to September 2011. Fifty-nine pre-transplant plasma samples were retrieved retrospectively. Overall, 27 patients were lost during the 24 months of the study period. The cohort included as many men as women, with a mean age of 48.3±1.5 years (mean±SEM; range: 21 to 83). Causes for end-stage renal dysfunction included diabetes (4% of the patients), vasculopathy (8%), glomerulopathy (22%), interstitial nephropathy (15%), uropathy (25%) or were not known (26%), in agreement with other cohorts (30). Fifteen and 2 patients had had one or two previous transplants, respectively. A large majority of the patients were under classical tri-therapy using Tacrolimus, Mycophenolate mofetil and steroids. Fifty-three patients received Basilixumab as adjuvant therapy (including 9 who also received IVIg) and 28 patients received anti-thymocyte globulins (including 16 with IVIg) and 18 patients did not receive any adjunct therapy. Ten patients received Rituximab alone (1 case), with Basiliximab (1 case), with anti-thymocyte globulins (7 cases) or with both (1 case).

IgG was purified and tested for hydrolysis of the peptide PFR-MCA, a surrogate substrate for catalytic antibodies with serine protease-like activity (16). The absence of contamination of the IgG samples by adventitious proteases was ensured by the use of a double-step purification procedure that involves a step of purification based on affinity and a step of purification based on protein size under denaturing conditions. Incubation of patients' IgG with PFR-MCA resulted in hydrolysis of the peptide and release of the

fluorescent MCA moiety. The released fluorescence allowed for the calculation of rates of hydrolysis. Hydrolysis of PFR-MCA was dose- and time-dependent (data not shown). Pooled IgG from healthy individuals demonstrated a marginal hydrolysis of PFR-MCA with an activity of 0.65±0.03 fmol/min per pmol (mean±SEM for 29 repeats). Irrespective of the time-point considered, IgG from renal-transplanted patients demonstrated significantly higher hydrolysis rates of PFR-MCA than pooled IgG from healthy individuals (Figure 1). The levels of PFR-MCA hydrolyzing IgG were extremely heterogeneous prior to transplantation, with a mean activity of 6.6±0.9 fmol/min/pmol (mean±SEM; coefficient of variation: 1.04, Figure 1). They decreased during the first three months that followed renal transplant to reach 2.4±0.2 fmol/min/pmol, and increased gradually during the 21 following months (3.2±0.3 and 5.1±0.6 fmol/min/pmol at 12 and 24 months post-transplant, respectively).

There was no significant difference in the levels of PFR-MCA-hydrolyzing IgG at any time point with respect to the sex, the age, the transplantation rank, the cause for end-stage renal failure, the time of dialysis prior to transplant, and the use of Basiliximab, anti-thymocyte globulins or Rituximab (data not shown and Table 1).

Treatment of patients with IVIg at the time of transplant is associated with a marked reduction of PFR-MCA hydrolyzing IgG 3 months post-transplant

Twenty-four patients (11 men and 13 women) had been treated with IVIg at the time of renal transplant. Treatment consisted in 4 cycles of administration of IVIg from the day of renal transplant and every three weeks thereafter. There was no difference between IVIg-treated patients and the remaining patients in terms of age, male to female sex ratio,

dialysis time prior to transplant and HLA mismatch score (Table 1). There was also no statistical difference in the distribution of the reasons for end-stage renal failure, although patients with uropathy tended to be more prevalent among IVIg-tretad patients. In contrast, and as expected, 47.8 % of the IVIg-treated patients had been transplanted previously as compared to 7.1% in the group of patients not treated with IVIg ($P<0.0001$). Likewise, 81.8% of the IVIg-treated patients had anti-HLA1 and anti-HLA2 antibodies, as compared to 48.2% for the remaining patients. Fifteen of the IVIg-treated patients received anti-thymocyte globulins and 8 received Basiliximab (one additional patient received both). There was no statistical difference in treatment protocols between IVIg-treated patients and patients not treated with IVIg (Table 1).

Patients treated and patients not treated with IVIg demonstrated similar rates of IgG-mediated PFR-MCA hydrolysis prior to renal transplant (6.1±1.0 vs 6.8±1.2 fmol/min/pmol, respectively, mean±SEM, Figure 1A). In contrast, the two groups of patients presented with statistically different rates of PFR-MCA hydrolysis by patients' IgG 3 months post-transplant: 1.5±0.3 fmol/min/pmol and 2.7±0.2 fmol/min/pmol for IVIg-treated and non-treated patients ($P=0.004$, using the two-tailed Mann-Whitney test). While both groups of patients experienced a decrease in catalytic activity during the first 3 months post-transplant, the decrease was more marked among IVIg-treated patients than in patients who did not receive IVIg: 4.0 vs 2.5-folds reduction, respectively. Levels of catalytic IgG were not different between the two groups of patients 12 months (3.5±0.6 vs 3.0±0.3 fmol/min/pmol, respectively) and 24 months (5.4±1.1 vs 5.0±0.7 fmol/min/pmol, respectively) post-transplant. Except in the case of the use of IVIg, there

was no difference in the levels of catalytic IgG according to the type of adjuvant immunotherapy (data not shown).

Levels of PFR-MCA-hydrolyzing IgG are not associated with the presence of anti-HLA antibodies

Because IVIg are generally administered to patients with high levels of anti-HLA antibodies in order to desensitize the patients (31), we investigated a possible bias between patients treated or not with IVIg with respect to the levels of PFR-MCA-hydrolyzing IgG. Patients were categorized based on the presence of either anti-HLA1 or anti-HLA2 antibodies, of both anti-HLA1 and anti-HLA2 antibodies or on the absence of anti-HLA antibodies. As is depicted in Figure 2, levels of PFR-MCA-hydrolyzing IgG were identical, irrespective of the presence or absence of anti-HLA1 and/or anti-HLA2 antibodies. This was true both prior to renal transplant and 3 months after transplant.

Discussion

In the present work, we investigated in an intermediate analysis the changes with time in levels of catalytic antibodies in patients receiving kidney transplants. To this end, we studied a large cohort of 100 consecutive patients followed in the same transplantation center. Patients included in the study were thus homogeneous in terms of clinical follow-up, treatment for prevention of graft rejection and origin of the drugs used in the treatment protocols. At the present time, data on CAN have not been obtained for all patients; CAN and rejection are thus not end-points of the present intermediate analysis.

The pathophysiological role of catalytic antibodies in the human is yet unclear. We and others had initially reported the presence of naturally occurring catalytic antibodies in human immunological and inflammatory diseases (8, 10, 11, 32), leading to the hypothesis that these antibodies may be deleterious. However, the description of catalytic IgM, IgG and IgA under physiological conditions (13-15), the finding that levels of PFR-MCA hydrolyzing IgG were elevated in patients surviving from septic shock (20), and the discovery in some patients with acquired hemophilia, a disease characterized by the presence of neutralizing anti-factor VIII autoantibodies, of factor IX-activating IgG (33), encouraged to revisit the initial hypothesis. Notably, we demonstrated in renal-transplanted patients that patients who did not develop CAN two years after transplantation had more elevated levels of PFR-MCA-hydrolyzing IgG (21). Our data highlighted the predictive value of measuring levels of catalytic IgG on the occurrence of CAN. The present work shows that patients with renal failure exhibit higher level of catalytic IgG than pooled normal IgG from healthy individuals (pre-transplantation data, Figure 1). It also reveals an extreme heterogeneity among patients in terms of level of

PFR-MCA-hydrolyzing IgG. The heterogeneity appears more as a general feature of pathological states than resulting from any particular cause for end-stage renal failure.

Our data document a reduction in the levels of catalytic antibodies after kidney transplant, followed by a progressive and slow restoration of the repertoire of catalytic IgG over time. Fluctuations in levels of catalytic IgG have rarely been studied earlier. Previous reports concentrated only on small numbers of patients in a retrospective manner, with low number of time points studied using serum or plasma that had not been systematically collected at pre-defined time points (28, 29). In contrast, one of the strength of the present study is the collection of plasma from a large number of consecutive patients in a prospective manner, and at different pre-determined time points: 3, 12 and 24 months following kidney transplant. Only the pre-transplant plasma samples were retrieved retrospectively. Of note, PFR-MCA-hydrolyzing IgG were tested at a constant IgG concentration for all the patients' samples (i.e., 67 nM). Hence, the changes in levels of catalytic IgG represent changes of the amount of catalytic IgG within the total IgG pool, and are thus independent from possible fluctuations of total IgG levels. Reasons for a drop in the levels of PFR-MCA-hydrolyzing IgG are probably linked to the immuno-suppressive treatment of the patients. Indeed, all patients received classical treatment using different combinations of steroids, cyclosporin, tacrolimus and/or mycophenolate mofetil. Furthermore, about 90% of the patients received adjunct immuno-suppressive therapy under the form of Basiliximab, anti-lymphocyte rabbit serum, Rituximab, plasmapheresis and/or IVIg.

In our cohort, 24 of 100 patients received IVIg therapy at the time of kidney transplant. Generally, IVIg is being used, either alone or in conjunction with plasmapheresis and/or

Rituximab, in patients undergoing transplantation across HLA barriers (24, 25, 31, 34-37). Thus, pre-conditioning patients "sensitized" to HLA with high-dose IVIg presents with short-term advantages, notably increasing transplantation rates, reducing the waiting time to transplantation and reducing acute humoral graft rejection. Accordingly, IVIg-treated patients in our cohort had significantly higher occurrence of previous graft and had a greater prevalence of anti-HLA1 and/or anti-HLA2 antibodies (Table 1). We observe here that the drop in the levels of PFR-MCA-hydrolyzing IgG 3 months following kidney transplant was statistically significantly more pronounced for patients treated with IVIg, as compared to patients who did not receive IVIg as adjunctive therapy. Mean levels of catalytic IgG in IVIg-treated patients were 55% that of the other patients. Jordan et al had previously documented a reduction of "panel reactive antibody" values following IVIg infusion to renal transplanted patients (24, 31). Although statistically significant in the studies by Jordan et al, the decrease in donor-specific antibodies among IVIg-treated patients was not as marked and consistent as the one we observe in the case of catalytic IgG.

Two explanations may account for the further reduction in levels of catalytic IgG 3 months post-transplant in IVIg-treated patients. The dose and timing of IVIg administration to the patients are compatible with a direct dilution effect of patients' IgG by the infused immunoglobulins. Indeed, considering that the patients received 2 g IVIg per kg body weight, that the average patient weight was 68 kg, that the blood volume may be estimated to approximately 5 liters, that the half-life of IVIg is 3 weeks (not considering the fact that some patients present with large proteinuria), and that the last administration of IVIg occurred 3 weeks prior to the 3-month sampling, one may broadly

estimate a 2 to 3-fold dilution of the endogenous IgG by IVIg, which incidentally corresponds to the observed further 2-fold reduction in levels of catalytic IgG. Alternatively, IVIg are endowed with immuno-regulatory effects on several innate and adaptive immune cells. In particular, IVIg have been shown *in vitro* to impact on B-cell proliferation, survival and differentiation, as well as modulate immunoglobulin secretion (38-44). Investigating putative changes in the levels of catalytic IgM might be a strategy to delineate between the two possibilities.

Our earlier work performed in a retrospective study that implicated 20 patients with kidney transplant, described that higher levels of catalytic IgG, both 3 and 12 months post-transplantation, were associated with a reduced incidence of chronic allograft nephropathy. The receiver operating characteristic curve derived from the levels of IgG-mediated PFR-MCA hydrolysis indicated that the hydrolytic activity of circulating IgG was a potential predictive marker for CAN, superior to the widely used biological parameters such as glomerular filtration rate (45) or proteinuria (46). The patients in the latter study had received similar immunosuppressive regimen as the patients included in the present cohort. Taken together, our data suggest that the reduction, associated with IVIg administration, in the levels of catalytic IgG in the patients 3 months post-transplant may jeopardize the use of catalytic antibodies as prognosis markers for chronic allograft nephropathy.

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Authors contributions

Designed the work : AM, AF, CL, OT, SVK, SLD

Performed the research : AM, IP, CD, BW, SPL

Contributed material : CL

Analyzed the data : AM, IP, LG, SPL, OT, SLD

Wrote the paper : AM, LG, SLD

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*

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Table 1. Characteristics of the study population

	IVIg-treated	**No IVIg**	**P value***
Number of patients	24	76	
Sex - M/F	11/13	39/37	
Age - years†	46.9±3.1 (21-73)	48.8±1.7 (22-83)	ns
Weight – kg	68.0±2.9 (51-109)	66.4±1.7 (39-114)	ns
ND	0	1	
Previous transplant - %	47.8	7.1	**<0.0001**
ND	1	6	
Cause of Nephropathy – Nb (%)#			
Diabetes	1 (4.2)	3 (4.0)	ns
Vascular	0 (0)	8 (10.5)	ns
Glomerulopathy	3 (12.5)	19 (25.0)	ns
Uropathy	10 (41.7)	15 (19.7)	0.056
Interstitial nephropathy	2 (8.3)	13 (17.1)	ns
Unknown	8 (33.3)	18 (23.7)	ns
Dialysis time prior to transplant - m†	65.2±9.4 (7-148)	50.2±5.5 (0-214)	0.091
ND	1	13	
HLA mismatch - score : 1 to 6 (range)	4 (0-5)	3 (0-6)	ns
ND	0	2	

Anti-HLA1 and anti-HLA2 Abs - %	81.8	48.2	**0.006**
ND	13	20	
Adjuvant immunotherapy - Nb‡			
Anti-thymocyte globulins	16	12	
Basiliximab	9	45	
Rituximab	7	3	
Plasmapheresis	2	3	
None	11		

* Two-tailed Mann-Whitney test; † Mean±SEM (range); ND : Not Documented; # Fisher's exact test

‡ All patients received Steroids, Cyclosporin, Tacrolimus and/or Mycophenolate Mofetil; one patient received both Basiliximab and anti-thymocyte globulins

Figure Legends

Figure 1. Treatment of renal transplant patients with IVIg is associated with a transient decrease in levels of PFR-MCA hydrolyzing IgG. IgG was purified from the plasma of patients who received IVIg therapy prior to transplantation (full circles) and from patients who did not received IVIg (empty circles). Plasma had been collected prior to renal transplant (D0) and 3 (M3), 12 (M12) and 24 (M24) months after renal transplant. IgG (66.67nM) was incubated with PFR-MCA (100 μM), a peptide chromogenic substrate, for 24 hr at 37°C. The amount of hydrolysis was quantified by measuring the fluorescence of the leaving MCA moiety, and is expressed in femtomoles of substrate hydrolyzed per minute per picomoles of IgG. IVIg was used as a control source of normal human IgG. Panel A depicts the raw results as scatter dot plots. Panel B depicts the evolution of the mean ± SEM levels of PFR-MCA-hydrolyzing IgG in the two groups of patients with time (*: P=0.004). The dotted line represents the hydrolysis of PFR-MCA by IVIg (mean of 29 measurements; Coefficient of variation: 0.29).

Figure 2. Levels of IgG-mediated hydrolysis of PFR-MCA among patients with anti-HLA antibodies. Patients were divided into three groups based on the presence of anti-HLA antibodies: no anti-HLA antibodies (circles); presence of either anti-HLA1 or anti-HLA2 antibodies (squares), and presence of both anti-HLA1 and anti-HLA2 antibodies (triangles). The graphs depict the rates of hydrolysis of PFR-MCA by IgG from each groups of patients purified from plasma collected prior to renal transplant (D0) or 3 months after transplant (M3).

Figure 1.

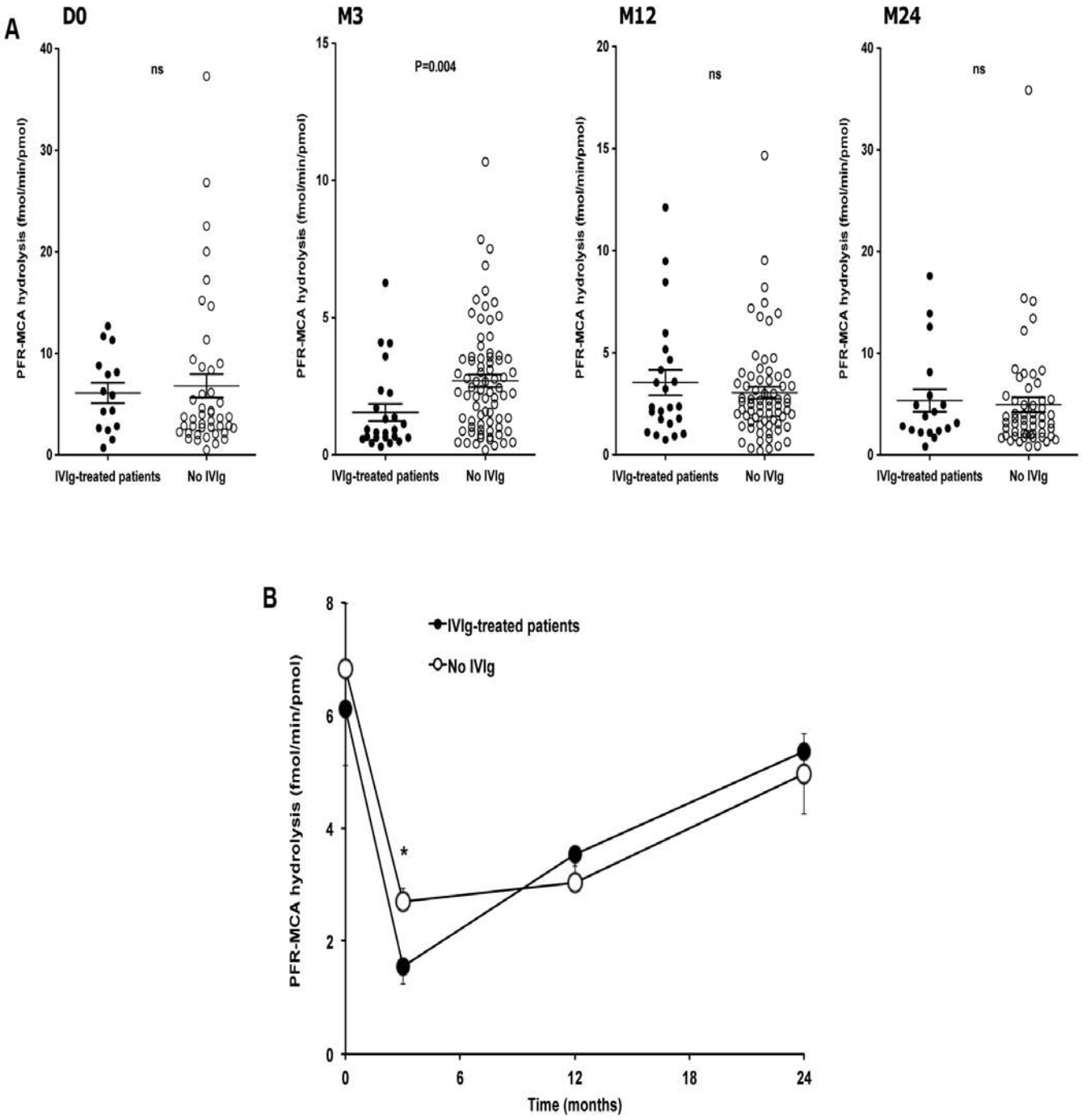
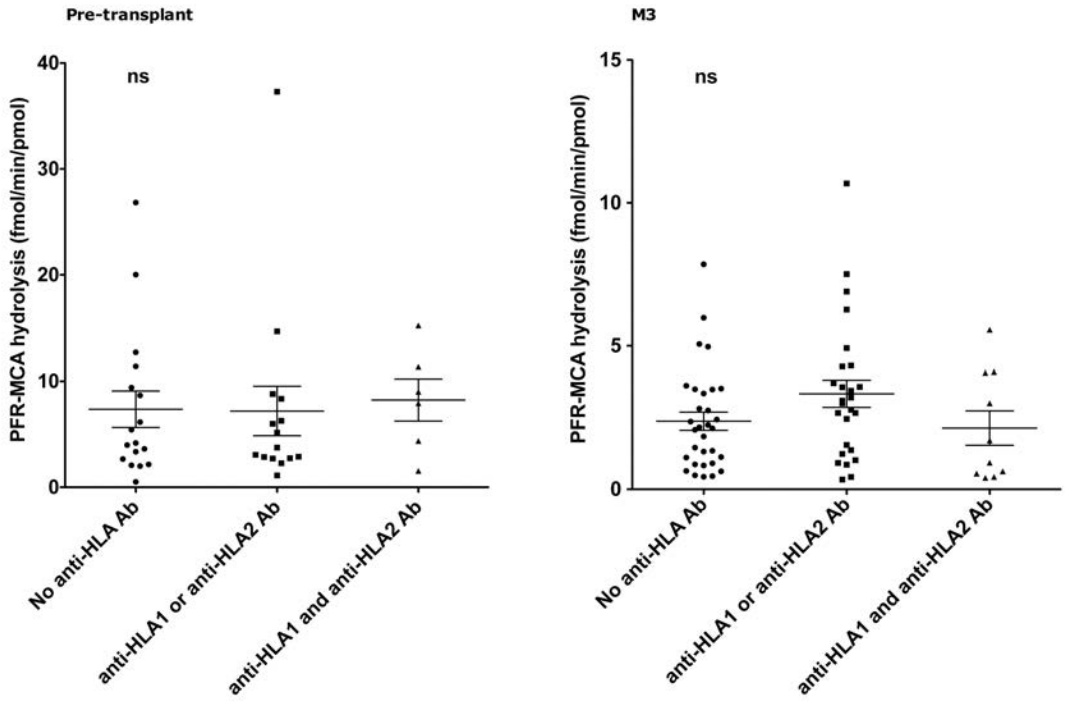

A
D0
M3
M12
M24
PFR-MCA hydrolysis (fmol/min/pmol)
ns
P=0.004
IVIg-treated patients
No IVIg
B
IVIg-treated patients
No IVIg
PFR-MCA hydrolysis (fmol/min/pmol)
Time (months)
0
6
12
18
24
*

Figure 2.


Pre-transplant
ns
PFR-MCA hydrolysis (fmol/min/pmol)
40
30
20
10
0
No anti-HLA Ab
anti-HLA1 or anti-HLA2 Ab
anti-HLA1 and anti-HLA2 Ab
M3
ns
PFR-MCA hydrolysis (fmol/min/pmol)
15
10
5
0
No anti-HLA Ab
anti-HLA1 or anti-HLA2 Ab
anti-HLA1 and anti-HLA2 Ab

Article 4- Résumé en Français

Titre: Importance de l'isotype des immunoglobulines dans la sensibilité à l'induction de polyréactivité par exposition à l'hème

Les immunoglobulines circulantes comprennent des anticorps dotés d'une spécificité unique envers leur antigène cible, appelés anticorps monoréactifs, et des anticorps capables de se lier à plusieurs structures antigéniques différentes et appelés anticorps polyréactifs. Une fraction des anticorps monoréactifs acquière des propriétés polyréactives après exposition in vitro à des agents redox tels que les ions ferreux, l'hème ou les dérivés réactifs de l'oxygène. L'injection d'IgG normales à un organisme inflammé induit la génération d'anticorps polyréactifs. Des études antérieures indiquent que, contrairement aux IgG polyclonales, les IgM polyclonales ne sont pas sensibles aux ions ferreux, alors que les deux préparations polyclonales sont sensibles à l'hème. Dans l'étude présente, nous avons étudié l'importance de la nature de la partie constante de la chaîne lourde des immunoglobulines dans la sensibilité à l'induction de polyréactivité. Nous avons isolé l'IgM circulante d'un patient avec lymphome B de la zone marginale de la rate. En parallèle, nous avons cloné les gènes codant les régions variables de l'IgM circulante sur un structure d'IgG. Les réactivités de l'IgM purifiée et de l'IgG recombinante ont été comparées avant et après exposition à l'hème par ELISA, dot blot, Western blot et 'surface plasmon resonance'. Les deux anticorps étaient également sensibles à l'induction de polyréactivité par l'hème. L'affinité à l'équilibre de l'IgM « induite » était 10 fois plus élevée que celle de l'IgG « induite ». Ces résultats contribuent à notre compréhension des mécanismes moléculaires en jeu dans l'induction de polyréactivité par les agents redox.

Title: Relevance of immunoglobulin isotype for sensitivity to polyreactivity induction upon exposure to heme

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Running title: Effect of Ig isotype on sensitivity to polyreactivity induction

Capsule:

1. Background: A fraction of circulating immunoglobulins acquires polyreactive behavior following exposure to redox agents, but the influence of the nature of the constant region on this effect remains ill-defined.

2. Results: The purified circulating IgM from a patient with splenic marginal zone B-cell lymphoma and the corresponding cloned recombinant IgG1 both demonstrated sensitivity to exposure to heme and acquire polyreactive behaviors.

3. Conclusion: The nature of the Fc portion does not dictate sensitivity to induction of antibody polyreactivity upon exposure to heme.

4. Significance: While the physiopathological relevance of the induction of polyreactive antibodies under oxidizing conditions remains unclear, our results suggest that all circulating antibodies that penetrate a pro-oxidative microenvironment may undergo alteration of their antigen-binding specificity.

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Word count :

Keywords : immunoglobulins, polyreactivity, heme, isotype

Summary

Circulating immunoglobulins encompass antibodies with single specificity for their cognate antigens, referred to as monoreactive antibodies, and antibodies with polyspecific binding behavior that are able to bind several structurally unrelated molecular targets. We and others have demonstrated that a fraction of the circulating monoreactive antibodies acquire polyreactive behavior following *in vitro* exposure to redox agents such as ferrous ions, heme or reactive oxygen species. Likewise, introduction of normal IgG into inflamed organisms results in the generation of polyreactive antibodies. Previous studies have indicated that, in contrast to normal polyclonal IgG, polyclonal IgM are not sensitive to ferrous ions but exhibit sensitivity to heme. In this work, we addressed the relevance of the nature of the constant part of the heavy chain of immunoglobulins for the sensitivity to induction of polyreactivity. We isolated the circulating IgM from a patient with splenic marginal zone B-cell lymphoma. In parallel, we cloned the V region genes encoding the circulating IgM into an IgG scaffold. The reactivity of the purified IgM and of the recombinant IgG was compared before and after *in vitro* exposure to heme by ELISA, dot blot, Western blot and surface plasmon resonance. Both antibodies demonstrated equal sensitivity to heme-induced polyreactivity. The equilibrium affinity of the “induced” IgM was 10 folds that of the “induced” IgG. The present results contribute to our understanding of the molecular mechanisms at play at the time of redox agent-induced antibody polyreactivity.

Introduction

Repertoires of immunoglobulins in healthy individuals represent an enormous diversity of antigen-binding specificities. This allows the immune system to generate high-affinity receptors specific for any potential antigenic structure. Based on their antigen-binding characteristics, immunoglobulins in a complete immune repertoire may be divided in two broad categories – monoreactive antibodies and polyreactive antibodies.

A polyreactive antibody is an antibody molecule that is capable of specific binding to several structurally unrelated molecular targets; these include proteins, nucleic acids, carbohydrates, phospholipids, peptides and haptens (1-3). Such antibodies represent a significant fraction (about 20 %) of healthy immune immunoglobulin repertoires. The genes encoding the variable regions of polyreactive antibodies are thought to be in germ-line configuration or to have a low number of somatic mutations (1,4). However, recent studies revealed that, in healthy individuals, a significant number of memory B cells express polyreactive B-cell receptors that have undergone affinity maturation and accumulate a high number of somatic mutations in the genes encoding their variable regions (5,6). These studies actually indicate that acquisition of polyreactivity by some immunoglobulins is the very result of the somatic hypermutation process. Polyreactive antibodies belong mostly to the IgM isotype, but polyreactive antibodies from other immunoglobulin isotypes (IgG, IgA and IgE) are also present (1,2). The interaction of polyreactive antibodies with multiple antigens is usually characterized by lower binding affinities (K_D values of 10^{-4}-10^{-7} M) as compared to the interaction of monoreactive antibodies with their cognate antigen (K_D values of 10^{-8}-10^{-11} M). The lower antigen-

binding affinity is compensated by enhanced functional affinity (avidity) in the case of decavalent IgM antibodies.

The molecular mechanisms underlying polyreactive antigen-binding are not completely understood. It was demonstrated that the binding of antigen by germ-line gene-encoded antibodies is accompanied by significant structural changes in their antigen-binding sites, especially in the CDR3 loop of the heavy chain (7-10). These antibodies demonstrated also high antigen-binding polyreactivity. Thus, the presence of a pliable antigen-binding site that can adapt to the molecular features of different antigens appears as the main trait of polyreactivity (11,12). However, there are case of polyreactive antigen-binding that are not accompanied by significant structural changes in the antibody paratope (13).

Different functions of polyreactive antibodies or polyreactive B-cell receptors have been proposed. The most important one could be a further diversification of the immune repertoires (11). Polyreactive antibodies can also participate in immune defense by establishing an innate-like first line of defense against pathogens (14,15). Interestingly, the concentration of such antibodies was found to increase in cases of some viral or bacterial infections (16,17). Moreover, some of the broadly neutralizing HIV antibodies show antigen-binding polyreactivity (18,19). The promiscuous antigen binding of natural IgM antibodies may contribute to the clearance of senescent or apoptotic cells or damaged macromolecules (20-22). Polyreactive antibodies were also proposed to exert immunomodulatory effects and, at the level of B cells, to participate in the maintenance of immune tolerance (1).

The above described polyreactive antibodies express their polyreactivity constantly from the time of their entry in circulation to the time of their catabolism. In addition to these

natural polyreactive antibodies, the normal immune repertoire contains a fraction of antibodies that can acquire antigen-binding polyreactivity post-translationally. Indeed, the temporary exposure of some monoreactive antibodies to conditions that can alter protein structure such as low pH, chaotropic agents or high-salt concentrations, results in acquisition of polyreactive antigen-binding characteristics (23-27). Usually, immunoglobulins are exposed to such conditions during their purification from plasma or cell culture supernatants. Thus, the phenomenon of induced polyreactivity could at least in part explain the different binding behavior that is observed in the immunoreactivity of antibodies when evaluated before and after purification from serum (28,29).

In addition to protein destabilizing agents that are used in laboratory practice for immunoglobulin purification, the induction of cryptic immunoglobulin polyreactivity occurs after exposure to agents that are present *in vivo* and can be released in large quantities at sites of inflammation and/or tissue damage. Indeed, *in vitro* and *in vivo* exposure of human immunoglobulins to reactive oxygen species, heme, ferrous ions or other pro-oxidative agents and conditions, have been shown to result in acquisition of polyreactive antigen-binding potential of antibodies (30-36). Cryptic polyreactive antibodies were detected in evolutionary distinct species such as human, mouse and chicken (31,33,37). Importantly, only a fraction of the antibodies from the normal Ig repertoire is sensitive to redox agents and the acquisition of polyreactivity occurs at concentrations of redox agents that are not influencing the overall Ig molecular integrity (38). Despite the potential biological importance of inducible antibody polyreactivity for the regulation of immune responses or as a first line of defense against pathogens (3), its molecular mechanism is not well understood. Thus, the molecular features that

distinguish monoreactive antibodies with cryptic polyreactivity from monoreactive antibodies or from naturally polyreactive antibodies are ill-defined. Likewise, the site on the immunoglobulin molecule that is affected by the redox agents, or whether the effect directly targets the antigen-binding site or residues outside the paratope remains to be elucidated.

Antibodies with different immunoglobulin isotypes are susceptible to induction of polyreactivity by redox agents. Thus, antibodies of the IgG, IgM and IgA isotypes may acquire antigen-binding polyreactivity after exposure to redox agents (32,33,39). However, these studies utilized antibodies from different isotypes that also differ in their antigen-binding specificity. Hence, the role of the constant immunoglobulin region on the susceptibility to induction of polyreactivity of antibodies that have identical variable regions is also not known. Recent studies have revealed the important role of the constant portion of immunoglobulin molecules for determining the affinity, specificity and functional activity of variable-region identical antibodies (40,41). In the present study, we explored the influence of the nature of the constant region of an antibody for induction of polyreactive binding. We generated a human monoclonal IgG antibody with identical variable region as a circulating monoclonal IgM isolated from a patient with splenic marginal zone lymphoma. We then compared the sensitivity to polyreactivity induction of the recombinant monoclonal IgG and purified monoclonal IgM, as well as the mechanisms underlying polyreactive antigen binding. The results contribute to our understanding of the molecular mechanisms of induced polyreactivity of antibodies, of the mechanism of functional pairing of variable and constant portions in immunoglobulins, and of the role of antibody valency in polyreactive antigen binding.

Material and methods

Sequencing of variable region gene sequences and gene analysis.

Bone marrow was obtained from a 57 year-old female patient (patient 47) with a circulating IgM diagnosed to have splenic marginal zone B-cell lymphoma, and after informed consent. Mononuclear cells were isolated by fractionation on Ficoll-Hypaque gradients (PAA, Velizy-Villacoublay, France) as described by the supplier. After centrifugation, the mononuclear cells ring was recovered and the cells were washed in PBS (Life technologies, Saint Aubin, France) and initially stored in liquid nitrogen with DMSO. For this study, cells were thawed rapidly and washed in PBS. High-molecular-weight DNA was extracted from mononuclear cells using the QIAamp DNA Mini Kit (QIAGEN, Courtaboeuf, France).

The rearranged variable genes encoding the heavy chain (V_H) and encoding the light chain (V_L) were amplified from genomic DNA (1 µg) using a mixture of forward primers annealing to the peptide leader region of the V_H and V_L genes, in combination with a single (for V_H) or mixture (for V_L) of primers specific for the joining genes as previously described (42-44). The PCR mixture contained 1x *Taq* buffer, 200 µM of each dNTP, 1.5 mM of MgCl2, 10%, 1 U of *Taq* polymerase (Ampli-Taq Gold, Life Technologies), and 0.2 µM of each primer in a final volume of 50 µl. PCR amplification consisted of an initial denaturation step at 94° C for 10 minutes, followed by 35 cycles at 94° C for 30 second, 64° C for 1 minute, and 72° C for 1 minutes, with a final extension step at 72° C for 10 minutes. All PCR reactions were performed using appropriate positive (clonal and polyclonal) and negative controls. PCR products were subsequently analysed either by GeneScan profiling on ABI 3730 DNA Analyzer (Applied Biosystems, California, USA)

for V_H rearrangements (using a fluorochrome-labeled IGHJ primer), or by standard polyacrylamide gel electrophoresis (for V_L rearrangements) according to the Biomed 2 protocols (44).

Monoclonal bands corresponding to V_H and V_L tumor gene rearrangements were excised from 1.5% low-melting agarose gels and the PCR products were purified from the gel with the QIAEX II kit (Qiagen). Thereafter, they were sequenced directly on both strands using the ABI 3730 DNA Analyzer (Applied Biosystems). Sequence data analysis, including identification of V, D and J genes as well as mutational status assessment, was done by comparing the tumor's V_H and V_L gene sequences to those of the corresponding human germline sequences presenting the highest homology using the IMGT® databases and the IMGT/V-QUEST tool (http://www.imgt.org).

Purification of the circulating IgM

The serum from patient 47 was subjected to affinity-chromatography on anti-human IgM agarose antibodies (Sigma-Aldrich, Lyon, France). The purity of the purified IgM was confirmed by SDS-PAGE and Western blotting.

Cloning, transfection and expression of variable region gene sequences.

The sequences of the variable region genes obtained from the bone marrow aspirates were re-synthesized commercially (Geneart AG, Regensburg, Germany). The restriction sites EcoR1 and Nhe1 were introduced in the sequence encoding the heavy chain variable gene (V_H) and restriction sites Age1 and Bsi W1 in the sequence of the light chain variable gene (V_L). The V_H sequence was cloned into the pFUSE expression vector

(Invivogen, Toulouse, France) that possesses a sequence encoding the constant region of the human IgG1 heavy chain. The V_L sequence was cloned into the pFUSE2 expression vector (Invivogen) that contains the sequence encoding the constant region of the human kappa light chain.

For expression of the recombinant antibody in eukaryotic system, human embryonic kidney cells (HEK-293) were used. Cells were first grown in HYPER flask (Corning, New York, USA) in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and non-essential amino acids (Life Technologies). The cloned expression vectors were mixed (350 µg of each) in 35 ml of 150 mM $NaCl_2$ and further treated at a ratio of 1:3with polyethleneimine (2.1 mg). The reaction mixture was let to stand for 10 min, was mixed in 500 ml of serum-free DMEM, containing non-essential amino acids and 1% nutridoma (Roche, Boulogne-Billancourt, France), and was added to the cells. Four days post-transfection, culture supernatant was collected and the recombinant IgG (rIgG47) were isolated by affinity chromatography on protein G sepharose (Amersham Biosciences, GE Healthcare Lifesciences, Velizy-Villacoublay, France). The purity of the IgG preparation was assessed on a 10% SDS-PAGE under reducing and non-reducing conditions.

Immunoreactivities of antibodies by ELISA.

Ninety-six well plates (Corning) were coated with human factor IX (10 µg/ml, Benefix; Baxter, Vienna, Austria), myelin basic protein (10 µg/ml, Sigma-Aldrich), human factor H (5 µg/ml, Complement Technologies Inc., Tyler, Texas, USA) and Apo-hemoglobin (10 µg/ml, Sigma-Aldrich). After 2 hr incubation at room temperature, the wells were

blocked with 0.2% PBS-Tween 20 (PBST). rIgG47 (100 μg/ml) was treated with hematin (0 to 16 μM) for 30 min on ice, and was diluted to a final concentration of 10 μg/ml prior to being incubated with antigen-coated plates for 2 hr at room temperature. Plates were washed and incubated with a peroxidase-conjugated goat anti-human IgG (Southern Biotech, Birmingham, Alabama, USA) for 1 hr at room temperature. Bound IgG were revealed by *measuring the absorbance at 492 nm after* addition of peroxidase substrate, *o-Phenylenediamine dihydrochloride (Sigma-Aldrich).*

Immunoreactivities of antibodies by Western blots.

Immunoreactivities on cross blots were investigated using cell lysates, including human endothelial cell (HUVEC), human liver and on recombinant human factor IX (FIX). Cell lysates (100 μg/ml) and recombinant antigen (10 μg/ml) were directly adsorbed on nitrocellulose membranes in individual slots of miniblotters 28 SL (Immunetics, Cambridge, MA, USA). After 1 hour incubation, the membranes were blocked with TBS-Tween 0.1%.

In the case of preparative Western blots, HUVEC lysates (100 μg) were electrophoretically separated on 10% preparative SDS-PAGE, and then transferred on nitrocellulose membranes. Membranes were blocked with TBS-Tween 0.1% overnight. The purified IgM and recombinant IgG from patient 47 (IgM47 and rIgG47) at 10 μg/ml were treated with varying concentrations of hematin (0 to 16 μM) for 30 min on ice. The membranes were then introduced perpendicularly in the miniblotters and the antibodies were incubated in individual slots. After 1 hr at room temperature, the blots were washed for 1 hr with TBS-Tween 0.1%. Fixed IgM and IgG were revealed using alkaline

phosphatase conjugated anti-human IgM or anti-human IgG antibodies (Southern Biotech, Birmingham, Alabama, USA) and the BCIP-NBT substrate (KPL Laboratories, Gaithersburg, Maryland, USA).

Binding of hematin to rIgG47 and IgM47 by absorbance spectroscopy.

The binding of hematin to IgM47 and rIgG47 was studied by UV-visible spectroscopy. Hematin was added at varying concentrations (0 to 6.4 μM) to IgM47 or rIgG47 (100 μg/ml in PBS, pH 7.4) and incubated in the dark for 5 min. Absorption spectra were measured at a wavelength range of 350-700 nm, with a scanning speed of 1500 nm/min. The absorption spectra of hematin alone were obtained at similar concentrations. The binding curves of hematin interaction with IgM47 and rIgG47 were obtained by subtracting the absorbance maxima in the Soret region of hemin in the presence of Ig by the absorbance maxima of hemin diluted in PBS alone ($A_{\text{heme-IgG}}$- A_{heme}) at 390 nm.

Surface Plasmon resonance analysis.

The kinetics of the interaction of hematin-treated IgM47 or rIgG47 with different antigens was measured by surface Plasmon resonance on a BIAcore 2000 (Biacore AB, GE healthcare Life Sciences). Myelin basic protein, Factor IX and Factor H were coupled to research grade CM5 sensor chip (GE Healthcare Life Sciences), as per the manufacturers instructions. Density of adsorbed antigens were 2.6, 1.8 and 5.0 ng/mm^2, respectively. IgM47 (111 μg/ml, 100 nM) and rIgG47 (100 μg/ml, 670 nM) were first treated with 8 μM of hematin in PBS for 5 min on ice. Hematin-treated rIgG47 and IgM47 were further diluted to 20 and 100 nM in HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% tween 20, pH 7.2) and injected in serial dilutions

on the immobilized antigens for 5 min at a flow rate of 10 µl/min at 25°C. The dissociation was then monitored for an additional 5 min. The surface of the chip was regenerated using 0.1 M glycine and 0.3% triton X-100, pH 12. The kinetic rate constants were calculated using the BIAevaluation version 4.1 software (Biacore).

Results.

Sensitivity of a recombinant IgG from a patient with SMZL to heme.

Patient 47 was characterized by a circulating IgM and by a monoclonal B-cell infiltrate in the bone marrow. DNA from the B-cell lymphoma was obtained by bone-marrow aspirate. The genes encoding the variable regions of the heavy (VH) and light (VL) chains of the monoclonal immunoglobulin were amplified and sequenced. VH and VL genes were then synthesized and cloned into human IgG1 and kappa expression vectors. The recombinant IgG (rIgG47) was produced in HEK293 cells and purified from supernatant by chromatography on protein G-Sepharose. We then investigated the reactivity of rIgG47 incubated alone or following exposure to hematin. rIgG47 demonstrated a marginal baseline binding to a series of self-antigens including factor IX (FIX), myelin basic protein (MBP), factor H (FH) and apo-hemoglobin (Figure 1), as well as factor VIII and calreticulin (data not shown). The binding of rIgG47 incubated with varying amounts of hematin increased in a dose-dependent manner. Two-hundred percents increase binding to self-antigens were obtained for hemin concentrations as low as 0.5 to 1 nM, i.e., a 7 to 15 molar excess of heme over IgG. Saturation of binding was obtained for hematin concentrations comprised between 4 and 10 μM, depending on the antigen. At saturation, IgG binding increased by at least 4-fold as compared to native rIgG47.

Sensitivity of rIgG47 and IgM47 to polyreactivity induction by heme.

Because rIgG47 was found to be sensitive to heme treatment, we decided to compare the capacity of both rIgG47 and of the circulating IgM purified from the plasma of patient 47

(IgM47) to become polyreactive following exposure to hematin. rIgG47 and IgM47 were incubated alone or in the presence of increasing amounts of hematin. Ig reactivity was tested with antigen extracts from HUVEC and human liver tissue (Figure 2A). Pooled human IgG and IgM from healthy donors were used as positive controls. As indicated in ELISA, rIgG47 demonstrated marginal autoreactivity with both tissue extracts in the absence of exposure to hemin; IgG reactivity increased in a dose-dependent manner with increasing heme concentrations. Interestingly, while native IgM did not react with both sources of self-antigens, it started binding following exposure to 0.5 μM heme. The binding intensity of IgM reached saturation at 2 μM heme, while that of IgG reached saturation at 5 μM heme.

We then compared the reactivity of rIgG47 and IgM47 with protein extracts of HUVEC separated in a preparative SDS-PAGE prior to transfer onto nitrocellulose membrane (Figure 2B). In both cases, there was a dose-dependent increase in the binding to HUVEC antigens with increasing heme concentrations.

Binding of heme to rIgG47 and to IgM47.

We applied steady-state absorbance spectroscopy in order to investigate the ability of heme to bind to rIgG47 and to IgM47. Steady state absorbance spectroscopy revealed changes in the UV-visible spectra of hemin upon incubation with Ig (Figure 3). The absorbance maxima in the Soret region increased when hemin was incubated in the presence of rIgG47 and IgM47, as compared to heme incubated alone. Changes in the low energy part of the spectrum (α and β regions of the spectrum: 500-600 nm) were also evidenced, suggesting a specific binding of the macrocyclic compound to the protein. The

absence of a significant shift in the Soret region of UV-visible spectra suggested that the binding of hemin to rIgG47 and IgM47 is not accompanied by the coordination of the central iron by amino-acid residues of the Ig molecules. Differential spectra analyses (insets) indicated that saturations of Ig with hemin were achieved above a 15-molar excess of hemin in the case of IgM47 and above 8 in that of rIgG47. This suggests the presence of approximately 10 binding sites for heme on both IgM47 and rIgG47. Fitting the data with a single binding site hyperbolic equation allowed calculation of the mean apparent affinity at equilibrium, which was equal to 2.5±0.2 nM in the case of IgM47, and 1.1±0.3 nM in that of rIgG47.

Kinetic parameters of the binding of heme-exposed IgM47 and rIgG47 to self antigens.

To obtain quantitative data about the influence of the Ig class on the polyreactive interactions of antibody 47, we used surface plasmon resonance-based real time interaction analyses. Different autoantigens were immobilized on sensor chips, including plasma-derived human factor H, human factor IX, bovine myelin basic protein and porcine tubulin. The real-time interaction profiles indicated that native IgG47 and IgM47 tested at 100 and 20 nM, respectively, did not bind to the immobilized proteins (Figure 4 and Supplement Figures S1, S2 and S3), thus supporting our data obtained by ELISA. Exposure of both Ab47 isotypes to heme resulted in considerable increase in the binding response (Figure 4 and Supplement Figure S1, S2 and S3). By applying global analyses to the interaction profiles, we evaluated the kinetic parameters of the interactions (See Table 1). The exposure of antibody 47 to heme resulted in acquisition of physiologically

relevant values of the binding affinity for different antigens. Thus, the determined values of equilibrium dissociation constant (K_D) for binding to different antigens for heme-exposed IgM47 and IgG47 ranged from 7.6 to 15.3 nM and 41.3 to 76.1 nM, respectively. The obtained values of the kinetic rate constants (Table 1) indicated that the relatively elevated values of binding affinity for the polyreactive Ab47 originate from high values of the association rate constant (Table 1), implying that the antibody-antigen complex is formed rapidly. The values of the association rate constant for the polyreactive IgM47 were marginally higher than that of the polyreactive IgG47 (Table 1). The values of dissociation rate constant for both IgG47 and IgM47 heme-exposed polyreactive antibodies were relatively high and in the same range, indicating a relatively poor stability of the complexes.

Further, we investigated whether the induction of polyreactivity by heme affects the binding of IgG47 to protein G. The real time interaction profiles of native IgG47 and heme-exposed IgG47 showed that both forms of the antibody bind strongly to immobilized protein G (Figure 5). Calculated binding affinities indicate that the binding of heme to the IgG molecule and ensuing transition to polyreactive antigen-binding behavior does not influence the binding affinity to protein G.

Discussion

In this work, we investigated the influence of the nature of the constant portion of antibodies in the sensitivity to induction of antibody polyreactivity by redox agents. To this end, we cloned the variable regions of the circulating IgM from a patient (patient 47) with a splenic marginal zone B-cell lymphoma after isolating DNA by bone morrow aspirate. The variable regions of the IgM were expressed on an human IgG1 scaffold. In parallel, we purified the IgM by affinity-chromatography from the serum of patient 47. We thus generated a recombinant antibody that has identical antigen-binding sites as the endogenous circulating IgM but different constant portions. The recombinant IgG and purified IgM forms of the antibody in their native states showed low binding activity to various autoantigens or to bacterial antigens. However, the exposure of the antibody to heme resulted in a dramatic increase in immunoreactivity. Antibody 47 acquired polyreactivity and started to recognize various self- and foreign antigens as evaluated by ELISA, immunoblot and Biacore binding analyses. The ability to acquire polyreactivity did not depend on the immunoglobulin isotype. The quantitative data obtained by kinetic analyses revealed that the polyreactive form of antibody 47 bind with relatively high affinity (K_D in low nM range, see Table 1) to all studied autoantigens. The high values of the association rate constants observed in the binding to antigens by both the IgG and IgM forms of antibody 47 indicated that the antigen-binding site of the polyreactive antibody form is pre-optimized for interaction with the protein antigens. The estimated binding affinity of IgM47 was 5 to 7-fold higher in case of all studied antigens as compared with the binding affinity of IgG47. This difference may be explained either by

the contribution of avidity effects in the case of a pentameric IgM molecule, or by effect of different isotypes on the configuration of the antigen-binding site (see below).

Accumulating evidence reveals the important role of the constant portion of immunoglobulin molecule for the fine-tuning of the antigen-binding properties of antibodies. It was demonstrated that the antigen-binding affinities of antibodies that possess identical V region sequences but different heavy chain isotypes differ significantly (45). Thus, the V region of the anti-tubulin antibody PER was expressed in an IgA and an IgG scaffolds. The binding affinity of monomeric intact IgA, $F(ab')_2$ IgA or Fab IgA fragments was considerably higher as compared to the binding affinity of the IgG variant (45). This observation highlights the modulation the antigen binding affinity of an antibody by the CH1 region of its heavy chain. The studies in the laboratory of Arturo Cassadevall clearly delineated the importance of the constant portion of immunoglobulins for determining antigen specificity and affinity. Thus, kinetic and thermodynamic analyses demonstrated that different IgG subclasses influence energetic changes and the overall affinity of an antibody against the Cryptococcus carbohydrate antigen (46,47). Moreover, this investigation revealed that the subclass of the heavy chain determinates the tendency for binding to multiple autoantigens, i.e., the polyreactivity of the antibody. The role of the nature of constant Ig regions in the antigen-binding properties of antibody paratopes was further confirmed by using a variety of biophysical approaches (48,49). It was proposed that the constant portion affects the antigen-binding site of antibody by long-range electrostatic interactions and conformational signaling through the VH-CH1 interface (40). Indeed, circular dichroism analyses during antibody interaction with cognate antigen revealed variations in the secondary structure in

antibodies that have identical variable regions but different constant regions (48). Moreover, NMR analyses demonstrated that the constant region of an antibody influences the electronic structure of the variable region and consequently its hydrolytic activity (49). The structural constraints exerted by different constant regions modulate the antigen binding sites and change not only the fine specificity and affinity of antibodies but also their functional properties (50). Thus, it was shown that the pathogenic potential of anti-DNA antibodies that possess the same variable regions strongly depends on the type of the constant region: recognition of DNA, binding to cells, binding to isolated glomeruli and potential to exert kidney-damaging effects strongly correlated with the Ig subclass and class (50). Another study revealed that the constant Ig region influences the functional activities of broadly neutralizing antibodies to HIV (51).

All these findings prompted us to investigate the role of the Ig constant region in determining the sensitivity to induction of antibody polyreactivity. Previous works demonstrated that cryptic polyreactive antibodies belong to different Ig classes – IgG, IgM and IgA (32,33,39). However, in these studies, Ig molecules with different variable regions were used. In the present work, we observed that, independently of the isotype, the exposure of antibody 47 to heme resulted in acquisition of polyreactivity. The breadth of the induced polyreactivity however may differ, which could be explained by the specific effect exerted by the different constant Ig regions on defining the fine specificity of antibodies. The sensitivity of both IgG and IgM isotypes to heme suggests that cryptic antibody polyreactivity is a property that depends on the interaction of the redox-active agents with the variable regions of the immunoglobulins. Our previous data had indeed demonstrated that heme induces polyreactivity by direct binding to the immunoglobulins

(34). Heme-binding restrains the conformational flexibility of the antibody paratope. Therefore, we hypothesized that antibodies exploit the inherent promiscuity of this compound as a cofactor for binding to multiple antigens (52). Here, absorption spectroscopy indicated that heme binds to both IgG47 and IgM47. The binding to both isotypes was characterized with similar spectral changes and the estimated binding affinities for heme were in a similar range (K_D values in low μM range). This result suggested that the binding of heme to cryptic polyreactive antibody is specific to the V region. The small differences in the affinity for heme to molecule with different isotype again may be explained by an influence of the constant portion on the fine specificities of antigen binding regions.

The interaction of antibodies with their cognate antigens may result in induction of conformational changes at long distance from the paratope. Indeed, a recent study demonstrated the presence of consistent structural alterations in the constant domain of various immunoglobulins upon antigen binding (53). This finding further supports the notion that the V and C regions of the immunoglobulins are functionally coupled. The binding of haptens by antibodies was shown to result in allosteric changes in the IgG molecule that modify its constant portion and hamper interaction with protein A and protein G molecules (54,55). In the present study, the kinetic data indicate that induction of polyreactivity by heme does not influence the binding affinity of IgG47 to protein G. This observation rules out putative heme-induced changes in the constant portion of the IgG molecule or heme-induced formation of IgG aggregates.

In contrast to the effect of heme on IgG, the exposure to ferrous ions – another agent previously demonstrated to reveal the cryptic antibody polyreactivity – did not induce

significant changes in the antigen-binding characteristics of either isotypes of antibody 47. This observation implies that cryptic polyreactive antibodies express differential sensitivities to different redox agents – a finding that can be explained by the existence of different mechanisms of induced antibody polyreactivity. Indeed, previous studies demonstrate that the mechanism of polyreactive binding by heme and ferrous ion-treated antibodies is different (33,34). The exposure of antibodies to ferrous ions results in an increase in the structural flexibility of the antigen binding sites. The increase pliability of paratopes may explain the better adaptability of the antibodies to multiple antigenic determinants. In contrast, heme rigidifies the antibody paratope and its binding seems indispensible for the antibody polyreactivity. Moreover, there are some differences in the sets of antigens recognized by heme and iron ion-induced polyreactive antibodies (56). Interestingly, our previous data indicated that pooled human IgM antibodies, in contrast to pooled human IgG, show only marginal sensitivity to induction of polyreactivity by ferrous ions (33). Taken together, these data indicate that revealing of cryptic polyreactivity occurs by different mechanism depending on the chemical nature of the redox agent.

Cryptic polyreactive antibodies have been shown to exert powerful anti-inflammatory effects *in vivo*. Thus, the treatment of animals with polyreactive heme- or ferrous ion-exposed IgG was found to protect from sepsis and autoimmune diabetes (33,57,58). In addition, cryptic polyreactive antibodies have been proposed as a biomarker for neurodegenerative diseases (59). The study of the molecular mechanisms underlying the cryptic nature of antibody polyreactivity is of uttermost importance to understand the physiopathological role of this antibody fraction and to explore its therapeutic potential.

The present study suggests that cryptic polyreactivity is a property dependent only on the unique amino-acid sequence and spatial rearrangement of the variable region; the constant region of the immunoglobulin molecule appears not to influence susceptibility to polyreactivity induction. However, based on the available literature, we can not rule out the possibility that the constant region participates in the fine-tuning of the repertoire of the recognized antigens and the binding affinities, once polyreactivity has been induced. Furthermore, whether induction of polyreactivity occurs spontaneously *in vivo* as a strategy to maintain homeostasis remains to be deciphered.

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Authors' contributions:

Designed the research: AM, SLD, JDD

Performed the research: AM, BG, CP, SA

Contributed indispensible material: FD, GML

Analyzed the data: AM, SLD, JDD

Wrote the paper: AM, SVK, SLD, JDD

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Table 1

Kinetic parameters of the binding of hematin-treated IgM47 and rIgG47 to self-antigens.

	IgM47			rIgG47		
Antigen	$\mathbf{k_{on}}$ x 10^4 $\mathbf{M^{-1}\,S^{-1}}$	$\mathbf{k_{off}}$ x 10^{-3} $\mathbf{S^{-1}}$	$\mathbf{K_D}$ x 10^{-9} M	$\mathbf{k_{on}}$ x 10^4 $\mathbf{M^{-1}\,S^{-1}}$	$\mathbf{k_{off}}$ x 10^{-3} $\mathbf{S^{-1}}$	$\mathbf{K_D}$ x 10^{-9} M
Factor H	53	5.7	10.8	5.8	4.0	69.3
Factor IX	75	7.0	9.4	10.7	4.7	43.8
MBP	56	4.3	7.6	7.2	3.0	41.3
Tubulin	50	7.7	15.3	8.6	6.5	76.1

Legends to figures.

Figure 1. Binding of hematin-treated rIgG47 to self-antigens by ELISA. Native or hematin-treated (0.25 to 16 μM) rIgG47 (10 μg/ml, 67 nM) were incubated with insolubilized factor IX (FIX), myelin basic protein (MBP), factor H (FH) and apo-hemoglobulin in ELISA plates. After washing, bound IgG were revealed using a HRP-coupled anti-human IgG antibodies and substrate. Data represents the mean absorbance ± SD of triplicate wells.

Figure 2. Binding of hematin-treated IgM47 and rIgG47 to self-antigens by immunoblot. Panel A. Cross blot analysis for immunoreactivities of native and hematin-treated (0.5 to 10 μM) IgM47 (left panel, 20 μg/ml) and rIgG47 (right panel, 20 μg/ml) with HUVEC and liver cell lysates. As a control, we used pooled normal IgM (IVIgM, 100 μg/ml) or IgG (IVIgG, 100 μg/ml) treated with hematin (0, 10, 20 μM). Intensity of dots was computed and mean values (±SD) are plotted versus the heme concentration and are expressed as arbitrary units. Panel B. A protein extract from HUVEC was separated on a 10% preparative SDS-PAGE. The binding of native and hematin-treated (0.125 to 16 μM) IgM47 (left panel, 20 μg/ml) and rIgG47 (right panel, 10 μg/ml) to HUVEC proteins was detected by Western blot. Immunoreactivities of native or hematin-treated (10 μM) IVIgM and IVIgG were analyzed as controls.

Figure 3. Binding of hematin to IgM 47 and rIgG47 by absorbance spectroscopy. IgM47 and rIgG47 (100 μg/ml) were titrated with hemin (0 to 6400 nM). Corresponding

aliquots of hemin were also added to a reference cuvette, containing buffer alone. The absorbance spectra of increasing concentrations of hemin alone (dashed line curves) and hemin in the presence of IgM47 (left panel, plain line curves) or of rIgG47 (right panel, plain line curves) were recorded in the range of 350-700 nm. Insets: the specific binding of hemin to IgM47 and rIgG47 was estimated by subtracting the absorbance maxima in the Soret region of hemin in the presence of Ig by the absorbance maxima of hemin diluted in PBS alone. Plotted data represent the excess of hemin over a concentration of Ig of 1 nM. Data were fitted using the single binding site hyperbolic equation (R^2=0.96 for both IgM47 and rIgG47, respectively).

Figure 4. Kinetics of interaction of native and hematin-exposed IgM47 and rIgG47 with MBP. IgM47 (20 nM, top panels) and rIgG47 (100 nM, bottom panels) were incubated alone (left panels) or with 8 μM hematin (right panels). The binding of the heme-IgM47 and heme-rIgG476 complexes to MBP was then measured in real-time by surface plasmon resonance using serial dilutions with initial Ig concentrations of 20 and 100 nM, respectively. The intensity of binding is depicted as resonance units (RU).

The binding profiles were fitted using the by BIAevaluation software (full red curves, Biacore AB).

Figure 5. Kinetics of interaction of native and hematin-exposed rIgG47 with Protein G. rIgG47 (100 nM) was incubated alone (left panel) or with 8 μM hematin. The binding of the heme-rIgG476 complexes to protein G was measured in real-time by surface plasmon resonance using serial dilutions with initial IgG concentrations of 100 nM. The intensity of binding is depicted as resonance units (RU).

Legends to supplemental data

Figure S1. Kinetics of interaction of native and hematin-exposed IgM47 and rIgG47 with factor H. IgM47 (20 nM, top panels) and rIgG47 (100 nM, bottom panels) were incubated alone (left panels) or with 8 μM hematin (right panels). The binding of the heme-IgM47 and heme-rIgG476 complexes to factor H was then measured in real-time by surface plasmon resonance using serial dilutions with initial Ig concentrations of 20 and 100 nM, respectively. The intensity of binding is depicted as resonance units (RU).

Figure S2. Kinetics of interaction of native and hematin-exposed IgM47 and rIgG47 with tubulin. IgM47 (20 nM, top panels) and rIgG47 (100 nM, bottom panels) were incubated alone (left panels) or with 8 μM hematin (right panels). The binding of the heme-IgM47 and heme-rIgG476 complexes to tubulin was then measured in real-time by surface plasmon resonance using serial dilutions with initial Ig concentrations of 20 and 100 nM, respectively. The intensity of binding is depicted as resonance units (RU).

Figure S3. Kinetics of interaction of native and hematin-exposed IgM47 and rIgG47 with factor IX. IgM47 (20 nM, top panels) and rIgG47 (100 nM, bottom panels) were incubated alone (left panels) or with 8 μM hematin (right panels). The binding of the heme-IgM47 and heme-rIgG476 complexes to factor IX was then measured in real-time by surface plasmon resonance using serial dilutions with initial Ig concentrations of 20 and 100 nM, respectively. The intensity of binding is depicted as resonance units (RU).

Figure 1

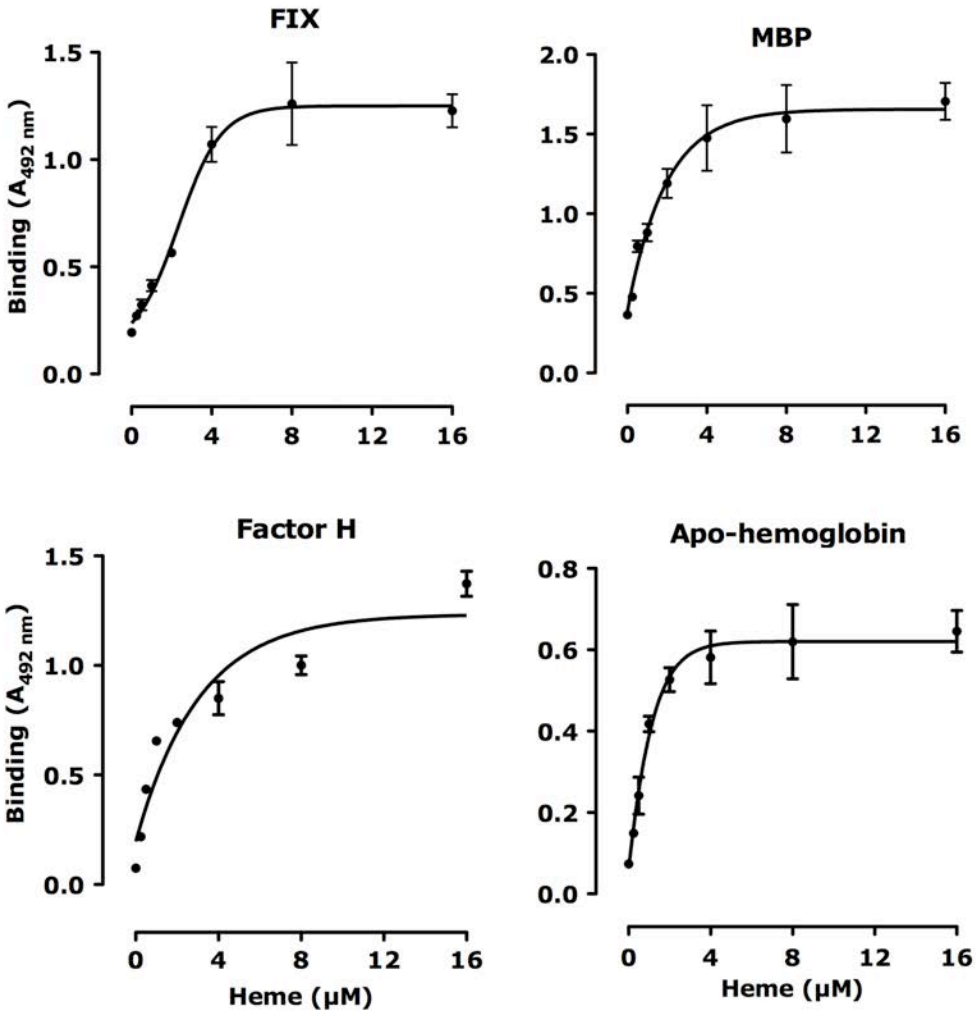
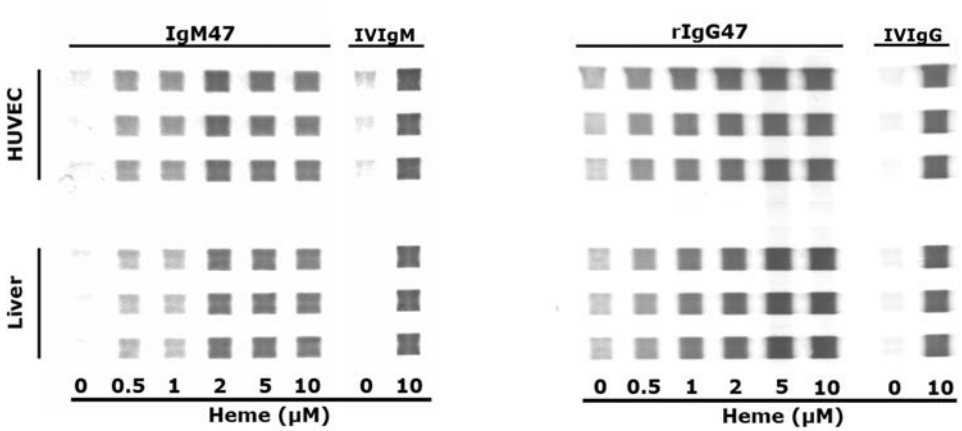
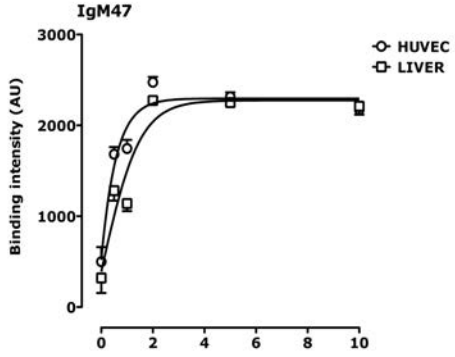

FIX
MBP
Factor H
Apo-hemoglobin
Binding ($A_{492\ nm}$)
Heme (μM)
0.0
0.5
1.0
1.5
2.0
0.2
0.4
0.6
0
4
8
12
16

Figure 2A


IgM47
IVIgM
rIgG47
IVIgG
HUVEC
Liver
0 0.5 1 2 5 10 0 10
Heme (μM)
0 0.5 1 2 5 10 0 10
Heme (μM)


IgM47
HUVEC
LIVER
Binding intensity (AU)
3000
2000
1000
0
0 2 4 6 8 10

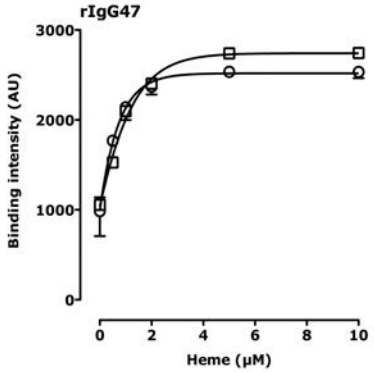

rIgG47
Binding intensity (AU)
3000
2000
1000
0
0 2 4 6 8 10
Heme (μM)

Figure 2B

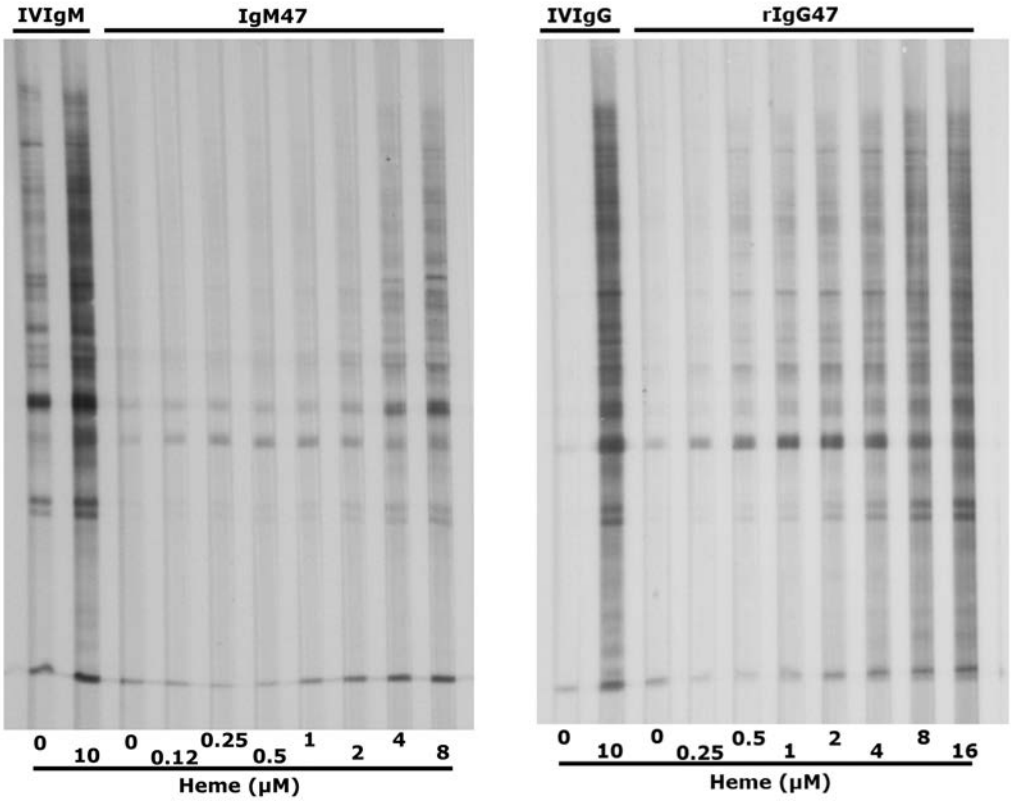


Figure 3.

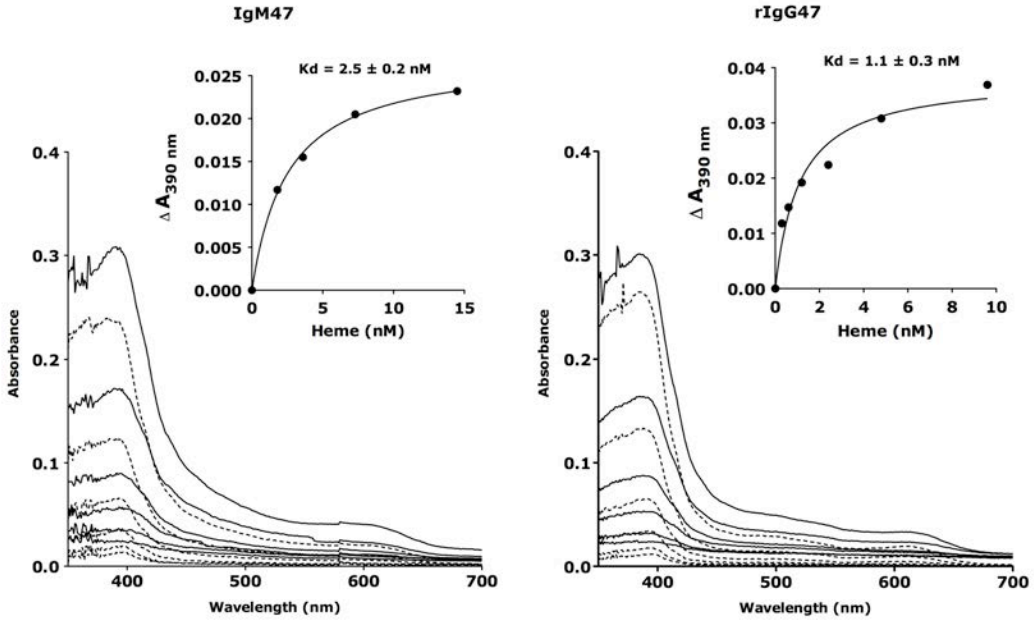


Figure 4.

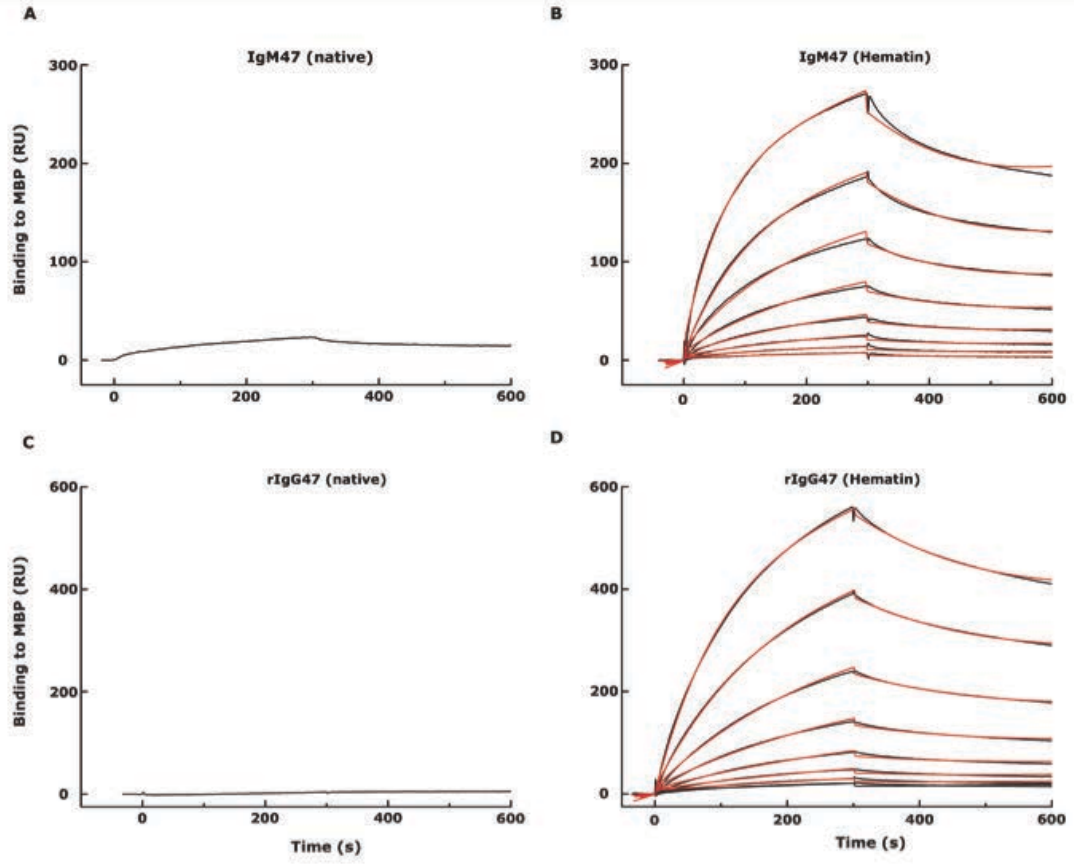


Figure 5.

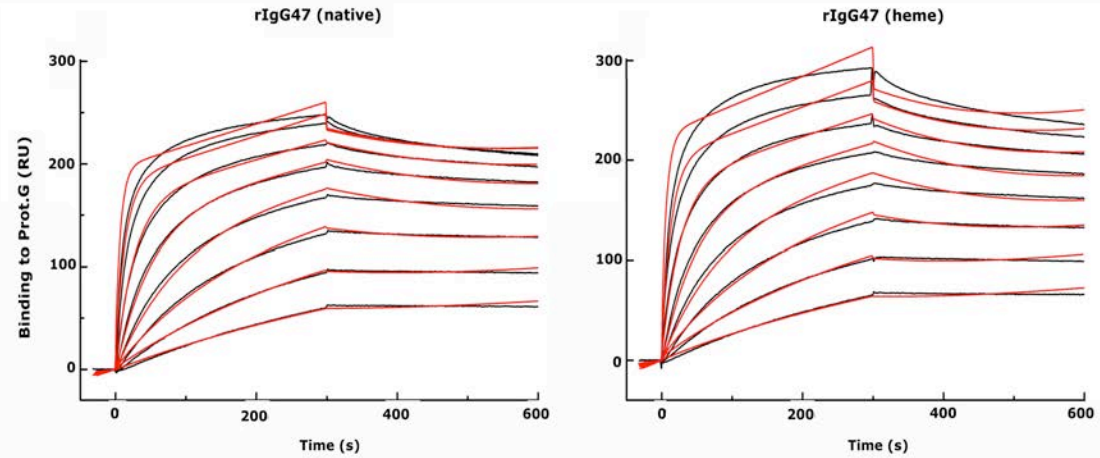


Figure S1.

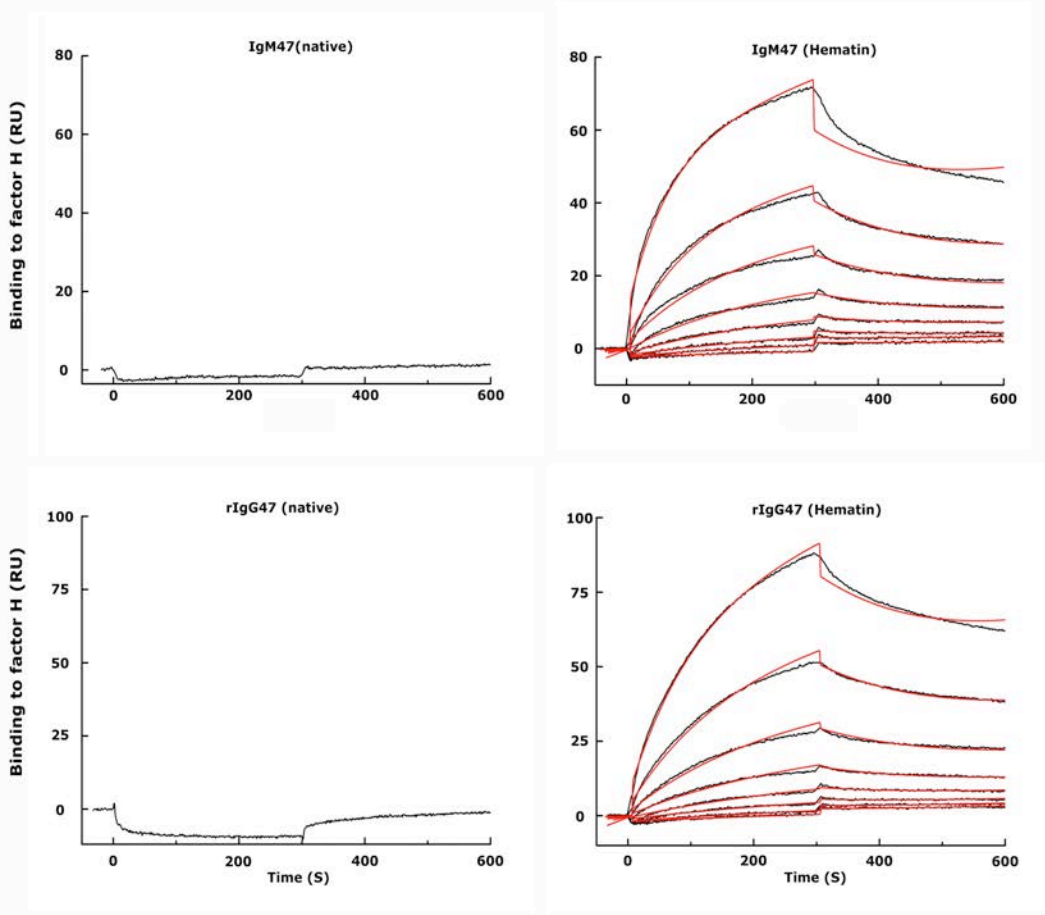

IgM47(native)
IgM47 (Hematin)
rIgG47 (native)
rIgG47 (Hematin)
Binding to factor H (RU)
Time (S)
0
20
40
60
80
0
25
50
75
100
0
200
400
600

Figure S2.

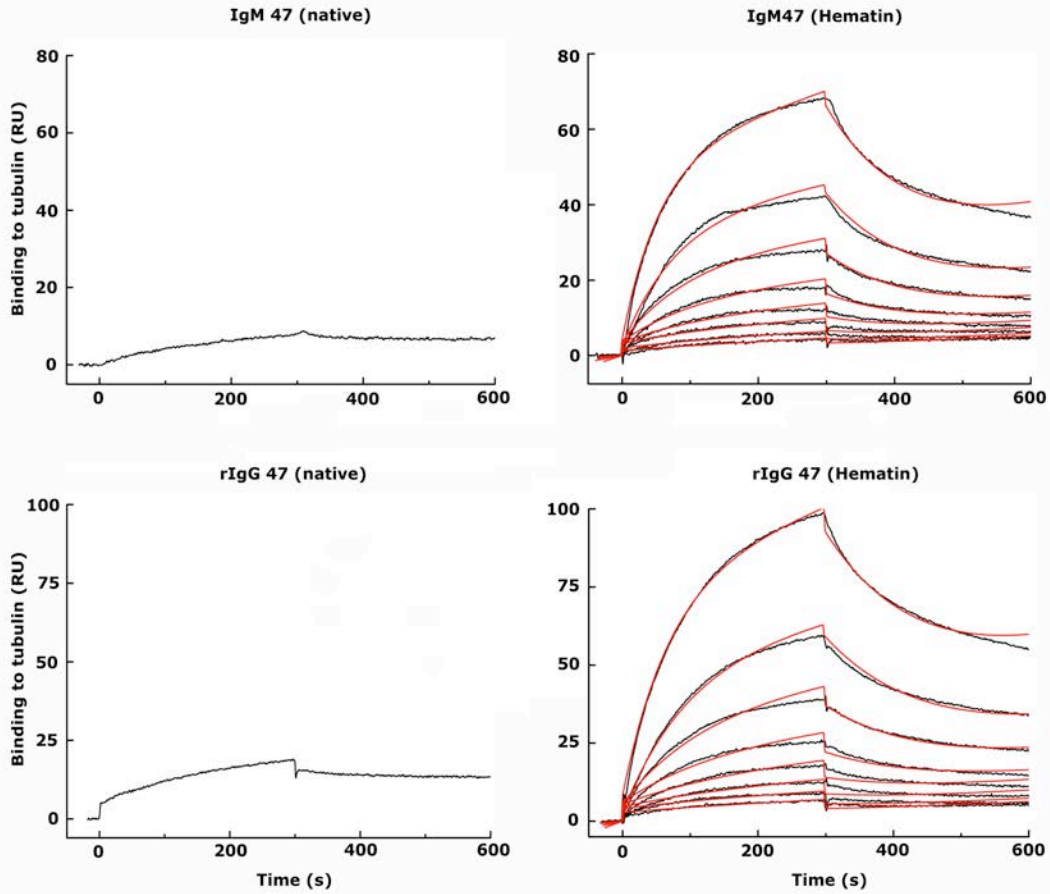
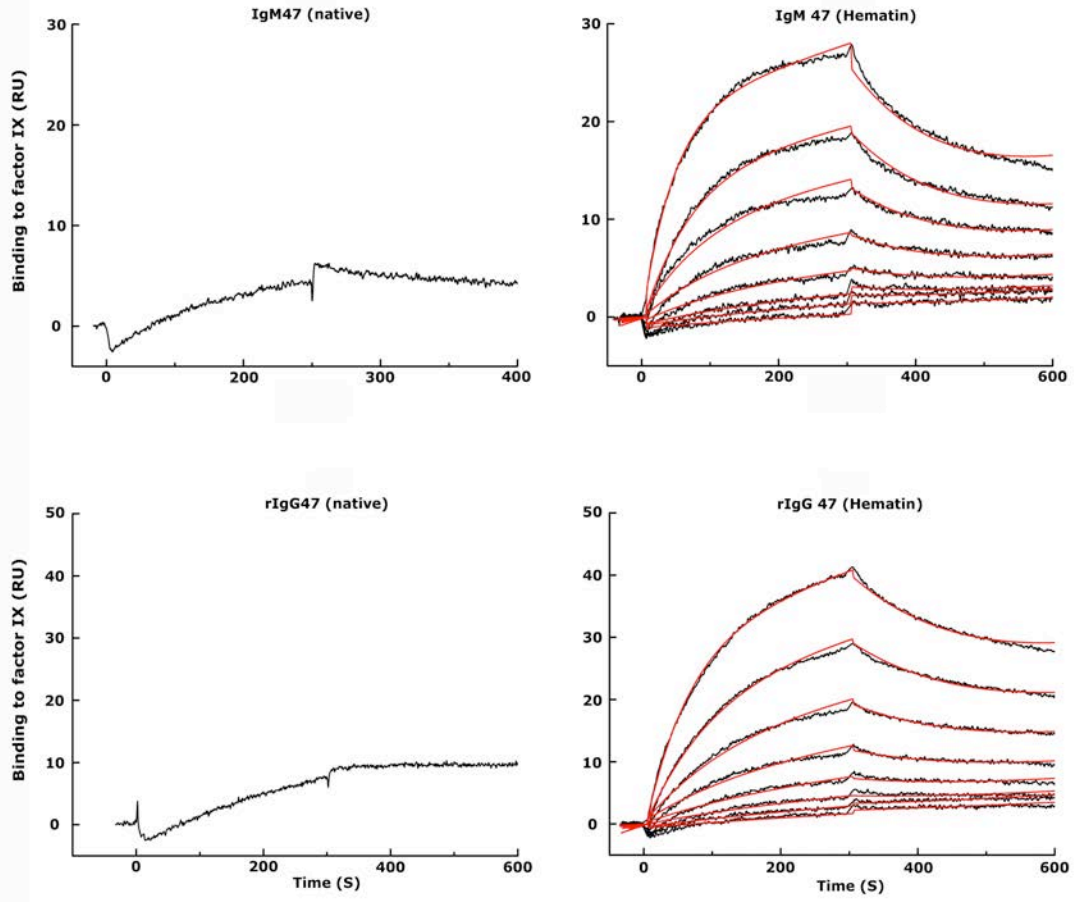

IgM 47 (native)
IgM47 (Hematin)
rIgG 47 (native)
rIgG 47 (Hematin)
Binding to tubulin (RU)
Time (s)
0
200
400
600
0
20
40
60
80
0
25
50
75
100

Figure S3.


IgM47 (native)
IgM 47 (Hematin)
rIgG47 (native)
rIgG 47 (Hematin)
Binding to factor IX (RU)
Time (S)

Discussion

Discussion

I my thesis work, I have focussed on two relatively less studied properties of antibodies. The first is antibody catalytic activity and second is polyreactivity. In this section, I will first discuss our observations on catalytic antibodies. This will then be followed by a discussion on polyreactive antibodies.

1. The origin of catalytic antibodies

The origin of catalytic antibodies had been a puzzling question ever since catalytic antibodies have been found in the human, that is in a situation when catalytic antibodies had not been "induced" upon deliberate immunization, but develop "spontaneously". The term "origin" covers actually two different aspects of the generation of catalytic antibodies: that of the genetic origin of catalytic antibodies, and that of their ontogenic origin: how are the B-cell clones producing catalytic antibodies generated during the ontogeny of the immune system and/or in the course of an immune response. In this first part of my discussion, I will concentrate on discussing the hypotheses proposed to explain the "spontaneous" presence of such antibodies in human beings.

1.A.Genetic origin of catalytic antibodies

The genetic origin of catalytic antibodies may be seen in two different ways. A first possibility is that some sets of genes, which are "dedicated" to encoding variable regions with a catalytic site, pre-exist in the gene repertoire of every individual. If this were true, it would mean that the catalytic function of antibodies has been evolutionary conserved -

if not selected for, a possibility that is justified by the fact that catalytic antibodies may be found in different species, such as human and mice. We believe that the lack of systematic research of spontaneously occurring catalytic antibodies in distant species such as chicken or sharks, prevents us from concluding on these lines. In collaboration with Dr Padiolleau-Lefevre, we performed an analysis of 23 monoclonal antibodies with amidase activity reported in the literature (Annexe. Article-1). Interestingly, our analysis pointed towards a high prevalence of catalytic antibodies using the rare antibody light chain variable gene subgroups IGKV2, IGKV9 and IGKV19 (Le Minoux et al., 2012). Another group headed by Dr Uda highlighted a preferential presence of serine protease-like catalytic triads in the antibody variable kappa gene subgroups bb1, cr1, cs1, bl1, bj2 and bd2 (Uda and Hifumi, 2004). It should be noted that both of these studies were based on the analysis of monoclonal antibodies that, by definition, result from the stimulation of the immune system, which thus does not exclude possible bias in the analysis. Of note, Uda et al identified catalytic triads with spatial arrangement of Ser-Asp-His similar to that found in the active site of proficient serine proteases (Hifumi et al., 2012), in the 3D structure of available monoclonal antibodies. They then cloned the corresponding germline genes and showed catalytic activity in the produced light chains. Thus, the light chains derived from germline genes A3/A19, A5, A17, A18, A23 of subgroup II, a relatively less expressed subgroup, was demonstrated to preferentially posses catalytic activity (Hifumi et al., 2012).

Another strategy to address the question of the origin of catalytic antibodies is to study the germline or mutated conformation of the genes encoding their variable regions. Historical studies in the 90's had compared the extent of germline mutations in induced

catalytic antibodies with esterase, oxy-cope rearrangement, Diels-Alder or VIPase activities (Table 1). Interestingly, the antibodies had been “demutated” to the germline configuration by site-directed mutagenesis. The catalytic activities of the germline and mutated antibodies had then been compared. The number of replacement mutations ranged between 3 and 14, which is not surprising given the fact that the antibodies had been induced by active immunization. Surprisingly however, there was no general rule as whether germline or matured antibodies are better catalysts or not. Indeed, the monoclonal antibody 48G7 was a better catalyst in the mature configuration, while clone AZ-28 displayed better catalytic efficiency in its germline configuration (Table 1). Conversely, clone C23.5 with VIPase activity displayed identical catalytic efficiencies, whether in its germline or matured configuration. In our review of the published data on catalytic antibodies (Annexe. Article-1), the variable regions of the heavy chains and light chains of the reported antibodies had more than 97% similarity with the corresponding germline genes, suggesting very low amount of somatic mutations. Mutations were more prevalent in the heavy chains than in the light chains, and light chains were more frequently involved in the catalytic process. Of note, in 50% of the cases, the mutation targeted an amino-acid that was presumed to be involved in the catalytic site.

As a conclusion of this part, it is clear that some “induced” catalytic antibodies are encoded by germline genes, which means that conserved amino-acids from the framework regions of the antibodies participate to the critical amino-acids of the catalytic site, while other “induced” catalytic antibodies result from a process of affinity maturation. In the latter case, whether the somatically mutated amino-acids are directly

part of the catalytic triad (in the case of antibodies with serine protease activity) or "shape" the 3D structure of the variable region to bring in close contact conserved amino-acids that make the catalytic site, remains to be determined. It is most probable that no general rule applies and that each case may be found. Nevertheless, it appears difficult to conclude with respect to the genetic origin of the catalytic antibodies that "spontaneously" develop on human beings, would it be under physiological or pathological conditions, because a systematic analysis of the variable region genes encoding these antibodies is still lacking, an might be difficult to achieve.

Table 2.

Clone	Activity	Nb replacements	Activity of Germline encoded	Activity germline vs mature	References
48G7	Esterase	9	+ (low)	germline <<<mature	Lesley 93, Patten 96
AZ-28	Oxy-cope rearragement	6	+	germline >> mature	Ulrich 97
39-A11	Diels-Alder	3	+	germline << mature	Romesberg 98
1E9	Diels-Alder	14	+	germline << mature	Xu 99
C23.5	VIPase	4	+	germline ≈ mature	Gao 94, Gololobov 99

1.B. Ontogenic origin of catalytic antibodies

The ontogenic origin of catalytic antibodies relates to the immune mechanisms that allow the production of B cells producing catalytic antibodies. A paradigm pointed out previously by S Paul is the necessity, for a B-cell clone to be selected, of a strong B-cell receptor (BcR) signalling. Contrarily, in the case of catalytic antibody-producing B cells, where the BcR is presumably catalytic and has a turnover, the binding stage of the BcR with the target antigen is very short since the antigen is catalysed and released. S Paul and others have thus derived strategies to "induce" catalytic antibodies, for instance using covalently reactive analogues (described in the introduction). The central question here however is the selection process of catalytic antibodies in an individual without deliberate immunization using "manipulated" immunogens. Hypothetically, the catalytic antibody response may be either antigen-driven, or may occur as a bystander response. In this second option, B cells possessing catalytic BCRs may spontaneously escape negative selection processes in bone marrow or at the periphery, or B cells may take advantage of a peculiarly low threshold of sensitivity for activation and benefit from ongoing immune activation in nearby microenvironment and be thus induced to produce catalytic antibodies. These hypotheses however remain unclear and require more investigation. In disagreement with the second option however, the generation of catalytic antibodies in physiology seems to be tightly regulated. Indeed, experimental data in autoimmune-prone MRL/lpr or SJL mice show a higher frequency of catalytic antibodies than in normal mice (Tawfik et al., 1995). Accordingly, protease and nuclease activity of antibodies from strains of autoimmune prone mice, MRL-lpr/lpr, NZB/NZW, SJL/J, have been reported to be very high in comparison to that of normal Balb/c mice. Of note, catalytic

antibodies of the IgM, IgA and IgG class, present in body fluids such as blood, saliva or mothers milk, have been reported (Semenov et al., 1998; Planque et al., 2004; Mitsuda et al., 2007). The presence of isotype switched catalytic antibodies again pleads in favour of a regulated generation of antibody catalysts.

S Paul and co-workers have shown the importance of the nature of the antibody constant heavy chain in the catalytic potency. Thus, data on human catalytic antibodies suggests that normal IgM express the highest catalytic activity, followed by IgA and IgG that are considered to be the least catalytic. The reason for IgM bearing the highest catalytic efficiency has been related to their tendency to be germline encoded. The germline configuration provides greater flexibility, relatively lower affinity for antigen and ultimately better turnover, properties that are a hallmark of efficient catalysts. Recent findings indicate that the constant domain plays an important role in the overall superior catalytic efficiency of IgM. Thus, variable regions cloned from a catalytic antibody were expressed in different scaffolds: single chain fragment variable (scfv), IgG and IgM: the recombinant IgM displayed the highest catalytic activity. In contrast, isotype switching (eg. from IgM to IgG), which is generally accompanied by somatic hypermutation, is a feature of mature humoral immune responses, and specializes antibodies towards efficient binding rather than turnover.

In humans, several autoimmune and inflammatory conditions are known for the sudden spontaneous generation of catalytic antibodies. Thus catalytic antibodies to self-antigens like vasoactive intestinal peptides (VIP), coagulation factor VIII (FVIII), DNA/RNA, myelin basic protein, thyroglobulin, and amyloid beta occur spontaneously in asthma, acquired and congenital hemophilia A, systemic lupus erythematosus, scleroderma,

multiple sclerosis, Hashimoto's thyroiditis and Alzheimer's disease. The reasons for an exacerbated generation of catalytic antibodies in diseases remains unclear. Given the complexity of some of the diseases in terms of the underlying conditions that causes the disease, it becomes difficult to identify the trigger that induces the catalytic antibody response. In this line, we recently tried to associate the levels of catalytic antibodies in patients receiving renal transplant, with the underlying diseases that were the cause for end-stage renal failure (Article 2). Among the different underlying conditions, that include diabetes, vasculopathy, glomerulopathy, uropathy and interstitial nephropathy, the lowest catalytic activity was found in patients with congenital defects (uropathy) as a cause for renal failure. Interestingly, kidney failure due to congenital defect is presumably not resulting from a global alteration of inflammatory, immune or cardiovascular processes. Besides, once treated by kidney replacement, patients with uropathy are not supposed to relapse. Taken together, our observation of higher levels of catalytic antibodies in renal transplant patients with diabetes, vasculopathy, glomerulopathy and interstitial nephropathy as compared to patients with uropathy is one additional piece of evidence that the trigger for higher levels of catalytic antibodies is associated with a dysregulated immuno-inflammatory status.

Under physiological conditions, catalytic antibodies have a promiscuous substrate specificity: they are generally revealed using substrates for amidases, such as the synthetic PFR-MCA peptide which we have used in our studies, and they fail to hydrolyze larger more complex antigens such as thyroglobulin, FVIII or FIX (Li et al., 1995; Wootla et al., 2008a). Whether the exacerbated catalytic antibody response in pathology is antigen-driven remains unclear. Our finding that the repertoire of catalytic

IgG changes with time in patients undergoing renal transplant, and acquire the capacity to hydrolyze FVIII (Article 2-3), is suggestive of a positive selection of the catalytic clones. Likewise, our finding of FIX-activating catalytic IgG in some patients with acquired hemophilia, but not in all patients, suggests an influence on the generation of catalytic B-cell clones, of the disease the pre-exists to the occurrence of anti-FVIII autoantibodies – a hypothesis not confirmed in our small cohort of patients. Alternatively, it may reflect the fact that patients wit the same disease are not equal in their capacity to mount a catalytic immune response.

2. The physiopathological relevance of catalytic antibodies

Historically, catalytic antibodies were studied by chemists and enzymologists. It is only in 1989 that catalytic antibodies were described for the first time in a context where the immune system had not been stimulated deliberately in order to boost the Ig catalytic response: spontaneously occurring IgG able to hydrolyze VIP were found in patients with asthma (Paul et al., 1989). Immediately, the catalytic activity of antibodies was proposed as a property to distinguish natural antibodies that circulate in physiological state from antibodies that are present in patients with different pathological conditions. Indeed, the list of diseases where catalytic antibodies are found extended rapidly from patients with Hashimoto's thyroiditis, to patients with systemic lupus erythematosus or with hemophilia A (Shuster et al., 1992; Li et al., 1995; Lacroix-Desmazes et al., 2002). These antibodies were also found to spontaneously arise in some animal models of human disorders (Ponomarenko et al., 2000). A general concept emerged according to which catalytic antibodies were associated with pathology, either as a hallmark of

immune dysfunction or as actors of the pathogenic processes. Simultaneously however, catalytic antibodies were described in the milk, blood and saliva of healthy individuals (Semenov et al., 1998; Planque et al., 2004; Mitsuda et al., 2007). In parallel, monoclonal human light chains endowed with catalytic activity were described in patients with Bence-Jones proteins (Paul et al., 1995) as well as monoclonal IgM in patients with Waldenström's macroglobulinemia (Planque et al., 2004). In the latter examples, it is probable that the catalytic activity was not responsible for the tumorigenic process and that the clonal catalytic light chains result from the expansion of spontaneously catalytic antibody-producing B-cell clones. A major difference between disease-associated catalytic antibodies and antibodies found in healthy donors was the specificity of the catalytic antibodies: while disease-associated antibodies hydrolyzed molecules related to the pathogenic process, i.e., VIP in patients with asthma, thyroglobulin in patients with Hashimoto's thyroiditis, FVIII in patients with hemophilia A, DNA in patients with lupus, naturally occurring catalytic antibodies had promiscuous amidase-like cleaving specificity and did not hydrolyze protein antigens.

The work which I have performed during my PhD, together with some of our anterior findings, opens a novel prospective as far as the physiopathological relevance of catalytic antibodies is concerned. Taken together, our work indicates that the levels of catalytic antibodies are always greater under the pathological conditions studied to date, that is hemophilia A (Lacroix-Desmazes et al., 2002), acquired hemophilia (Wootla et al., 2008a), sepsis (Lacroix-Desmazes et al., 2005), renal transplant (articles 2 and 3), than in healthy donors. In most of the cases, higher levels of catalytic antibodies are associated with positive outcome form the disease – this was demonstrated in sepsis, in chronic

allograft nephropathy (Wootla et al., 2008b), and suggested in acquired hemophilia (Wootla et al., 2011) (article 1). The closest case where catalytic activity could have been claimed to be pathogenic, i.e., anti-FVIII IgG in allo-immunized patients with congenital hemophilia A, has recently been revisited by Grosbois et al – the authors have depicted FVIII-hydrolyzing IgG in patients without anti-FVIII allo-antibodies, and an inverse evolution of the levels of catalytic antibodies and of anti-FVIII IgG in patients treated by high dose FVIII to induce specific tolerance (Grosbois et al.). More importantly, we demonstrate that catalytic IgG that hydrolyze and activate FIX are able to restore some levels of thrombin generation *in vitro*. Our current hypothesis is that, for reasons which are yet unclear, the B cells that produce catalytic antibodies are activated under pathological conditions. Such an activation could result either from a loss of negative control as proposed by Tawfik (Tawfik et al., 1995) or from a positive selection by a yet unknown antigenic trigger. It is tempting to speculate that the increased levels of circulating antibodies represent an attempt of the unbalanced organism to re-establish the homeostasis of the immune system. It could however simply be that increased levels of circulating catalytic antibodies are a hallmark of inflammation/hyper-immunity as it is the case for C-reactive protein (CRP).

3. FVIII as a preferential target for catalytic antibodies

Since the late 80s, scientists have investigated the presence of catalytic antibodies against a plethora of disease-associated target molecules. The results of most of the studies indicated that indeed, catalytic antibody response in the diseases was associated to the antigen that is central to pathology of the disease, for example, catalytic antibodies against thyroglobulin in Hashimoto's thyroiditis or amyloid beta in the case of Alzheimer's. We were the first group that demonstrated the generation of catalytic antibodies in an alloimmune response to the exogenous FVIII in patients with congenital hemophilia A. Factor VIII hydrolysis by purified IgG was observed in the case of 54 percent (i.e. 13 of 24) of patients with severe congenital hemophilia A. Later, we also observed the presence of FVIII-hydrolyzing catalytic autoantibodies in patients with acquired hemophilia. In the study cohort of 45 patients, 21 (47%) patients' IgG displayed significant hydrolysis of FVIII. Growing evidences however suggest that the presence of FVIII-hydrolyzing catalytic antibodies is associated in other diseases where FVIII is not directly implicated in the pathogenesis. In this line, we have earlier reported the presence of catalytic antibodies to FVIII in patients with sepsis and in patients that have undergone renal transplant. Thus, our observations indicate that the context in which FVIII-hydrolyzing antibodies emerge is far more complex.

The clues to understand the different context, in which FVIII-hydrolyzing antibodies may be generated, could be found through the structure or function of FVIII. Structurally, FVIII is considered to be an inherently unstable molecule. In addition, the stability of FVIII in vivo depends upon it's binding to various plasma proteins or antibodies, proteolytic-inactivation and non-proteolytic degradation. The occurrence of FVIII-

inhibitory antibodies has been proposed to be due to the increased immunogenicity that occurs because of the presence of the FVIII degradation products. The question however arises as, why does FVIII become a target for catalysis in diseases whose pathogenesis is not even distantly related. The probable answer lies in the central role played by FVIII in coagulation and/or coagulation-induced inflammation. In the coagulation cascade, FVIII plays an important role as a co-factor for activated FIX (FIXa) and FX, thereby increasing the velocity of FX activation by FIXa by several thousand folds. Interestingly, there are ample evidences of an extensive cross-talk between coagulation and inflammation. The importance of this cross-talk has been reported in several thrombotic complications. Indeed, one of the major causes of complication in sepsis is disseminated intravascular coagulation. In addition, in patients with renal transplant, activation of the stressed endothelium results in the amplification of the coagulation cascade - a major cause of chronic allograft nephropathy.

These observations suggest that due to the central role played by FVIII in coagulation and inflammation, it is possible that the inflammatory microenvironment favours an anti-FVIII immune response that includes generation of catalytic antibodies to FVIII. It can also be inferred that catalytic antibodies against FVIII can be deleterious or beneficial depending on the type of disease they are associated with. Thus, FVIII-hydrolyzing antibodies in hemophilia can be deleterious, however, in prothrombotic conditions such as sepsis or CAN the presence of catalytic antibodies against FVIII may be beneficial.

4. Factor IX-hydrolyzing IgG in different diseases

Our initial work on FVIII-hydrolyzing IgG in congenital hemophilia A failed to detect hydrolysis of other self-antigens, such as human serum albumin and human recombinant FIX used as controls (Lacroix-Desmazes et al., 2002). However, IgG-mediated hydrolysis of FIX was detected, together with FVIII hydrolysis, in one of the patients who survived from severe sepsis (Lacroix-Desmazes et al., 2005). In the case of CAN, FIX hydrolysis was observed by IgG of 3 patients. No satisfactory explanation could be provided at the time, although it is possible that the combined hydrolysis of FVIII and FIX exerts anti-thrombotic effects and participates in preventing disseminated intravascular coagulation, thus protecting the patients.

Recently, we have found that IgG from some patients with acquired hemophilia may hydrolyze FIX in addition to FVIII. The proteolytic activity was a property of the antigen binding site, as it was recovered with the purified $F(ab')_2$ fragments of the patients' IgG. Of note, FIX-hydrolyzing IgG were not detected in inhibitor-positive patients with congenital haemophilia B. Taken together, these data suggest that, in contrast to FVIII-hydrolyzing IgG that are found in congenital as well as acquired hemophilia, the spontaneous generation of FIX-hydrolyzing IgG requires the endogenous presence of the antigen. Interestingly, the migration profile of IgG-hydrolyzed FIX resembled that of activated FIX. Indeed, N-terminal sequencing of the IgG-mediated FIX degradation products confirmed cleavage of FIX at the arginine 180 residue and we confirmed that IgG-mediated hydrolysis of FIX lead to the activation of the coagulation factor (Wootla et al., 2011). Overall, IgG from 38.5% of the patients included in the cohort were able to activate FIX. IgG-mediated FIX activation tended (P = 0.07) to be significantly

associated with patient survival 12 months following diagnosis. In vitro assays showed that the FIX-hydrolyzing IgG were able to generate an average 0.3 nmol/l of activated FIX per 24 h. Such a modest amount of activated FIX was able to restore thrombin generation *in vitro*, provided that a basal level of FVIII ≥3% was present in the assay (Wootla et al., 2011). Although the measured kinetics of IgG-mediated FIX activation are low when compared to that of physiological FIX-activating enzymes (i.e., activated factor XI), we believe that the low kinetics are compensated by the long half-life and high concentration of IgG in the blood. Moreover, a fraction of the circulating FIX upon IgG-mediated activation may feed in the coagulation cascade and compensate for the loss of FVIII activity in patients with acquired hemophilia.

5. Catalytic antibody in renal transplant

A large amount of work during my thesis has been dedicated to studying the evolution of catalytic antibodies in patients undergoing renal transplant (Articles 2 and 3). My interest was motivated by the earlier finding of the laboratory that circulating levels of catalytic IgG may predict chronic allograft rejection (CAN), thus opening the way towards translational research and potential use of catalytic antibody detection in clinical practice (Wootla et al., 2008b).

While initiating the project on catalytic antibodies in renal transplant, I was initially pursuing two original objectives. The first one was to confirm the predictive value of the presence of catalytic antibodies on CAN in a prospective study involving a large number of patients. Conclusions on this end have not been reached as yet, since all data on CAN

are not available for the 73 patients that remain following two years of follow-up. Hence, the results presented in this thesis are that of an intermediate analysis.

The other objective that I was interested in was less clinical and more fundamental. Very few studies had concentrated on following the evolution with time of catalytic antibodies in human beings. The only studies were performed by our research group, either in small patient cohort such as patients with septic shock (Lacroix-Desmazes et al., 2005), or in large cohorts of patients with rare diseases, such as acquired hemophilia (Wootla et al., 2008a), a disorder that is extremely heterogeneous owing to the variety of underlying diseases that pre-exist to the development of the anti-FVIII autoantibody, to the clinical manifestations and to the treatments used. Both in septic shock and in acquired hemophilia, it was hardly possible to get blood or serum from the patients before the disease was diagnosed, and it was thus very complicated to relate heterogeneous levels of catalytic antibodies to any particular underlying disorder. Unlike most of the studied diseases, the incidence rate of renal transplant is relatively high and the patients are routinely followed-up for the evaluation of the graft functions. In collaboration with Ivan Peyron, another PhD student of the laboratory, I thus collected in a prospective manner the blood from 100 consecutive patients 3 months after they underwent kidney transplantation. Blood was also collected 12 and 24 months post-transplantation. Unfortunately, plasma samples from only 59 patients could be retrieved retrospectively. The questions which I wanted to address where as follows:

. Is there heterogeneity in the circulating levels of catalytic IgG in patients? Is associated with particular underlying pathological conditions? Is it intrinsic to each individual's immune system?

. Levels of catalytic IgG have been shown to vary with time (Wootla et al., 2008a). Is it possible to evaluate the fluctuations of catalytic IgG in a systematic manner? Can we conclude on the stability or plasticity of the "catalytic B cell" repertoire?

. How do the immunosuppressive treatments dedicated to preventing acute graft rejection affect the repertoire of B cells that secrete catalytic antibodies?

. Our earlier study had shown hydrolysis of FVIII and FIX by patients' IgG. Is the antigen/substrate-specificity of the catalytic IgG pre-determined or is it generated in the course of the disease?

5.A. Heterogeneity in the circulating levels of catalytic IgG in patients with end-stage kidney failure

Previous studies from the laboratory had brought to important results (Lacroix-Desmazes et al., 2002; Lacroix-Desmazes et al., 2005; Wootla et al., 2008a; Wootla et al., 2008b): human beings in general had exacerbated levels of circulating catalytic IgG under pathological conditions; different patients with the same pathology present with heterogeneous levels of catalytic IgG. Indeed, in patients with end-stage renal failure, and prior to kidney transplant, the levels of catalytic IgG were significantly higher than that of pooled IgG from healthy donors (Article 2). Besides, as observed for patients with congenital hemophilia A, with acquired hemophilia and with septic shock, the circulating levels of catalytic IgG were extremely heterogeneous prior to transplant. We failed to find an association between the levels of catalytic IgG and age or sex (Article 2). This indirectly suggests that levels of catalytic IgG are not gender-specific. It also suggests

that levels of catalytic IgG are not fluctuating in a systematic manner with ageing. There was also no association with number of previous transplants or time of dialysis prior to transplant. A lack of association with the number of previous transplant is compatible with the fact that the patients recover, 24 months after transplant, similar levels of catalytic IgG as that measured prior to transplant (Article 2). It does not say however whether the actual repertoire of target antigens have been altered or preserved.

When we compared patients with different causes for end-stage renal failure, it became apparent that patients with uropathy tended to have lower levels of catalytic IgG than the other groups of patients, even though the differences were not statistically significant owing to the low number of cases in some of the groups (in particular, diabetes, vasculopathy) (Article 2). Interestingly, patients with uropathy often develop nephropathy due to congenital reasons, and most often due to physical abnormalities. Once the kidney has been replaced, nephropahty is not supposed to relapse. In contrast, the other disease groups are characterized by inflammatory conditions, vascular problems or autoimmune manisfestations. Taken together, the data suggest that dysregulation fo the immuno-inflammatory status may lead to a flare up of catalytic Ig-producing B-cell clones.

5.B. Fluctuations of circulating levels of catalytic IgG

There was a drastic decrease in the IgG-mediated hydrolysis of PFR-MCA at 3 months post-transplant (Articles 2 and 3). Of note, IgG from all patients at all time points were tested for catalytic activity at a constant IgG concentration, hence the catalytic activity

observed at different time points was not due to the fluctuations in the total IgG concentration but was a reflection of the levels of catalytic IgG in the plasma.

The cause of the decrease in IgG-mediated catalytic activity is certainly linked to the immunosuppressive therapy. Indeed, all patients received classical treatment using different combinations of steroids, cyclosporin, tacrolimus and/or mycophenolate mofetil. Furthermore, about 90% of the patients received adjunct immuno-suppressive therapy under the form of Basiliximab, anti-lymphocyte rabbit serum, Rituximab, plasmapheresis and/or IVIg (Article 3). Assessment of the effect of steroids on the decrease of IgG-mediated catalytic activity was not possible, as almost all patients were administered with at least one form of steroid. However, separation of patients into groups based on the use of different adjunct therapies revealed a significant exacerbated decrease in catalytic activity in patients who were administered with repeated doses of IVIg at the time of transplantation. The effect of IVIg on the decrease of IgG-mediated catalytic activity may be linked either to a dilution effect of the patients' endogenous IgG with the administered exogenous IVIg, or due to the direct immunomodulatory effects of IVIg on the hosts' immune effectors (Article 3).

Follow-up of the patients revealed a significant recovery of the levels of PFR-MCA hydrolyzing IgG at 12 months, followed by a further increase at 24 months as compared to that at 3 months. The recovery after 24 months was substantially high as indicated by the absence of significant difference in the mean rates of IgG-mediated PFR-MCA hydrolysis in comparison to that of pre-transplant. While the present data do not allow to conclude on the plasticity or stability of the repertoire of catalytic antibodies under physiological conditions, or in an "un-manipulated" diseased organism (because the

patients are treated with immunosuppressive drugs), the data clearly plead for intrinsic capacity of the patients' immune system to express catalytic antibodies at higher than physiological levels. In other words, our data suggest that, when fluctuations on the levels of catalytic antibodies are imposed on the organism (by drugs), then the organism replenishes the depleted pool of catalytic antibodies. Evolution of levels of circulating catalytic antibodies may thus be envisaged as a complex process: under physiological conditions, B cells secreting catalytic antibodies are controlled and their expression is repressed to minimal levels; under some pathological conditions, most probably inflammatory or autoimmune, the B cell clones are activated in an individual dependent manner – some patients have the intrinsic capacity to mount a strong "catalytic immune response" (without presuming of its role, significance, efficiency), other patients mount only marginal catalytic immune responses; the levels of circulating catalytic IgG are then stable, and are re-established if eliminated by transient immuno-suppressive treatments – which may be due to the chronic stimulation of the "catalytic immune system" by a yet unidentified disease-related trigger, or to the fact that the repressor mechanisms that were at play under physiological conditions have been perturbed and cannot be re-established.

5.C. Is the antigen/substrate-specificity of the catalytic IgG pre-determined or is it generated in the course of the disease?

Chronic allograft nephropathy (CAN) is one of the major causes of late stage renal transplant failure. Deregulated activation of the coagulation cascade due to the uncontrolled activation of the endothelium drastically impairs graft function and may

result in total loss of function due to increased fibrosis of the graft (Matsuyama et al., 2003). The coagulation molecule FVIII plays a central role in the amplification loop of thrombin generation. Our previous observation in CAN and sepsis documents that the IgG mediated hydrolytic activity is directed against coagulation FVIII and/or FIX (Lacroix-Desmazes et al., 2005). Indeed, IgG-mediated hydrolysis of PFR-MCA correlated with that of IgG-mediated FVIII hydrolysis in patients with CAN. Thus, circulating hydrolyzing IgG may exert their preventive effect against CAN by disrupting the thrombin amplification loop. In the present study, we have observed that FVIII hydrolyzing IgG evolves with the general evolution of catalytic activity. The rate of FVIII hydrolysis by pre-transplant patients' IgG is high, however, no correlation is observed between the IgG-mediated PFR-MCA and FVIII hydrolysis. After transplant, IgG-mediated PFR-MCA hydrolysis tends to correlate with FVIII hydrolysis at three months and shows a significant correlation at 12 months. Thus indicating that, in the course of the disease, there is a change in the repertoire of catalytic IgG, which is shifting towards FVIII specificity. The reason for this shift towards FVIII specificity is unclear but it is tempting to speculate that it depends on the changing coagulation status of the surrounding endothelium of the graft.

6. Role of constant domain in heme-induced polyreactivity

Antibody molecules can be divided structurally into a variable and a constant domain. The constant region mediates the functions of antibodies by two major mechanisms that are: antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Conversely, the variable regions impart their function by binding to

the epitopes on antigen or sometimes through enzyme-like catalysis. For a long time, the two domains of the antibodies were considered mutually exclusive of each other in terms of the functions they perform. However, growing evidences now show that the constant region plays an important role in tuning the fine specificity and sensitivity of the binding of variable regions to their cognate antigens (Pritsch et al., 1996). Additionally, changes in the subclass of antibodies have been associated with a tendency towards polyreactivity, resulting in binding to multiple autoantigens (Torres et al., 2007; Dam et al., 2008). Among the domains within the constant region (CH_1– CH_4), it is the CH_1 domain that is considered to be responsible for imparting this effect (Pritsch et al., 1996). Interestingly, a recent study describes a huge increment in the antibody-mediated catalytic activity when cloned in an IgM scaffold as compared to its IgG or scfv counterparts (Sapparapu et al.). Thus, antibody constant regions may impart conformational changes in the variable domains, thereby modulating the antigen-binding functions. Indeed, long range interaction between the VH and CH1 region have been proved experimentally to structurally influence the antigen-binding site (Torres and Casadevall, 2008).

In the case of induced polyreactive antibodies, the modulation of variable regions by protein destabilizing agents is well known. While there is data on the induction of polyreactivity in antibodies with different isotypes (IgG, IgM, IgA) upon treatment with protein destabilizing agents, the results do not truly state the influence played by the constant region, due to the presence of different variable regions in each of the antibody isotypes studied (Omersel et al.; Dimitrov et al., 2006; McIntyre et al., 2006). Hence, it remains unclear whether changes in the constant domain may influence the polyreactivity induced by protein destabilizing agents. In this respect, one of the aims of my thesis was

to look at the role played by the constant region in heme-induced polyreactivity. For this, variable regions obtained from a monoclonal B cell of a patient with splenic marginal zone B cell lymphoma (SMZL) was cloned into an IgG scaffold. The binding profile and kinetics of interaction with various self and non-self antigens were then compared between the circulating IgM isolated from the plasma of the patient and the corresponding recombinant IgG. Our data suggest that the change in isotype does not modulate the specificity of heme-induced binding to different antigens, however the affinity of the variable region in the IgM scaffold for all antigens was approximately 10 times higher. The higher affinity of the IgM may be due to the avidity effect posed by the pentameric structure of IgM. In view of these results, it might be appropriate to compare the monomeric form of the IgM with that of the recombinant IgG. The IgM isolated from plasma showed a small amount of impurity with IgG when evaluated by ELISA and Western blot. To eliminate a possible bias in our results by the contaminating IgG, I have now cloned the variable heavy chain genes in an expression vector containing the constant region of IgM. Presently, I am in the process of selecting stable clones of recombinant IgM-secreting HEK293 cells, which have been simultaneously transfected with expression vectors containing the light and heavy chains. The monoclonal preparation of IgM will contain largely pentameric but also monomeric forms of the recombinant IgM. We will separate the pentameric and monomeric forms through size-exclusion chromatography and further evaluate the difference in heme-induced polyreactivity of the two forms of recombinant IgM and recombinant IgG.

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A novel molecular analysis of genes encoding catalytic antibodies

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ABSTRACT

Among the numerous questions remaining opened about catalytic antibodies (abzymes), the understanding of the origin of the genes encoding them is of vital significance. An original statistical analysis of genes encoding abzymes is described in the present report. Results suggested that these genes display a high conservation degree with their germline counterpart and a limited number of amino acid changes. Hence, on the contrary with high-affinity antibodies, maturation process by accumulation of somatic hypermutations is not required for the catalytic function. We demonstrated that despite a weak somatic mutation rate, the physicochemical properties of mutated amino acid (AA) are predominantly dissimilar with that of the germline AA. Further, we developed a novel approach in order to analyze the nature of genes encoding catalytic antibodies. For the first time, an unexpected and significant high level expression of rare gene subgroups was noticed and emphasized. The data described in this paper would lay the foundation for future studies about origin of genes encoding catalytic antibodies.

1. Introduction

Catalytic antibodies are immunoglobulins that are able to accelerate a chemical reaction. Originally, catalytic antibodies were generated by procedures using transition state analogues (TSA) as immunogens (Pollack et al., 1986; Tramontano et al., 1986). Since then, a hundred of antibodies with catalytic activity have been produced by using optimized strategies based on TSA or alternative ways (Xu et al., 2004). Catalytic antibodies were also found in human beings in the absence of deliberate immunization. Indeed, the presence of catalytic antibodies has been described in the serum of healthy individuals (Kalaga et al., 1995; Kit et al., 1996; Paul et al., 2004; Barrera et al., 2009) and patients suffering from autoimmune diseases (Shuster et al., 1992; Ponomarenko et al., 2002, 2006; Wootla et al., 2008, 2011) as well as other pathological conditions (Belogurov et al., 2009). This universal presence of abzymes raising the question of the mechanisms at stake that mediate the selection of the B cell clones producing antibodies with a catalytic propensity.

Analysis of somatic hypermutations of catalytic antibodies by comparison to the closest germline genes has been performed on an individual case basis (Sharma et al., 2009; Zein et al., 2010a,b). However, this did not provide a general overview of the mechanisms implicated to get catalytic features. Hence, it remains unclear whether the repertoire of variable (V) genes encoding catalytic antibodies is similar to or different from that encoding binding antibodies. Studies are required to define the role of somatic hypermutation in relation with a catalytic function. In addition, rearrangements of V, diversity (D) and joining (J) genes are necessary for the synthesis of the VH domains (V–D–J), and rearrangements of V and J genes for the synthesis of the VL domains (V–J) of the light chains kappa or lambda (Lefranc and Lefranc, 2001). Thus, the role of specific V–D–J or V–J rearrangements and the pairing of Vl and VH in the acquisition of the catalytic activity have to be clarified. Answering to these questions is complicated because of the presence of catalytic antibodies both under physiological and pathological conditions (Belogurov et al., 2009). Though there are numerous reports on the characteristics of catalytic antibodies, several questions are

Abbreviation: TSA, transition state analog.

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still unanswered. For example, whether the rate of mutation in the genes encoding catalytic antibodies is less or more or equal as compared to the genes encoding non-catalytic antibodies? Are there specific gene subgroups expressed by catalytic antibodies as compared to non-catalytic ones? Answers to these questions could help to understand the mechanism of generation of catalytic antibodies.

In this paper, systematic evaluation of the sequences of the catalytic antibodies referred in literature has been made. We have collected all available sequences of antibodies displaying the ability of breaking the amide bonds. We focused on amidase activity for the following reasons: (i) the catalytic amino acids required for such an amidase activity are well characterized, (ii) the hydrolysis of the amide bond is performed by antibodies that have been associated with physiological and pathological states in human beings. In several reports, studies of genes encoding catalytic antibodies of human or murine origin are based on a single technique (either by immunization or by phage display selection) (Wedemayer et al., 1997; Yang and Schultz, 1999; Paul et al., 2006; Zein et al., 2010a,b). In this report, irrespective of the techniques, we dealt with catalytic antibodies not only of mouse origin but also from human donors (Table 1). By this way, the bias of production of antibodies by a single technique was minimized.

Herein we have focused on both the extents of somatic hypermutations and the nature of the V genes used to encode catalytic antibodies. In particular, on the basis of the germline analysis, we have evaluated (i) the rate and the nature of somatic hypermutations expressed by the catalytic antibodies considering the estimated role of the amino acid (AA) changes in catalysis, (ii) the frequency of the immunoglobulin (IG) V gene subgroups that encode catalytic antibodies in comparison with those encoding binding antibodies. A robust statistical analysis was performed to study the frequency of genes encoding catalytic antibodies in two separate sets. First, sequences of murine catalytic antibodies specifically displaying amidase activity were analyzed. Second, the studies were also extended to the antibody sequences of both human and murine origin, regardless of the type of catalytic activity.

2. Materials and methods

2.1. Gene analysis

The published V domain sequences of antibodies displaying the ability of breaking amide bonds were collected: the latter reactivity is shared by several enzymatic activities, including protease, peptidase and β-lactamases (Table 2). Gene analysis was further extended to catalytic antibodies endowed with other activities, i.e. DNAse, esterase, decarboxylase, transaminase and Diels-Alderase (Table 2). Nucleotide sequences and translations were available from the GEDI nucleotide databases (GenBank (http://www.ncbi.nlm.nih.gov/genbank), EMBL-Bank (http://www.ebi.ac.uk/embl), DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/), and IMGT/LIGM-DB (http://www.imgt.org). Amino acid sequences from three-dimensional (3D) structures were from the RCSB Protein Databank (PDB) (http://www.rcsb.org/pdb) and from IMGT/3D structure-DB (http://www.imgt.org). Sequences from antibodies i41-7 and HpU-9-L have been obtained from T. Uda (personal communication), and FT6 and FT12 were from the corresponding publication (Tanaka et al., 1999).

The germline V and J genes of each sequence were identified and analyzed by using the IMGT® databases and tools (IMGT®, the international ImMunoGeneTics information system® http://www.imgt.org) (Lefranc et al., 2009). When nucleotide sequences of V domains were available, we used the IMGT/V-QUEST tool (Brochet et al., 2008; Giudicelli et al., 2011) to identify the closest germline V and J genes and to determine the percentage of nucleotides identity between the V domain and the germline sequences. When only amino acid sequences were provided, we used the IMGT/DomainGapAlign tool (Ehrenmann et al., 2010; Ehrenmann and Lefranc, 2011). The consistency between the two tools was controlled by submitting the nucleotide sequence to IMGT/V-QUEST and the corresponding amino acid sequence to IMGT/DomainGapAlign for several antibodies. In each case, both tools suggested the same germline genes.

2.2. Evaluation of the physicochemical properties modifications due to the AA changes

Amino acid changes in strands and loops were analyzed according to the IMGT/DomainGapAlign tool (Ehrenmann et al., 2010; Ehrenmann and Lefranc, 2011). This tool allows to estimate the physicochemical impact of AA changes due to mutations. Eleven IMGT 'Physicochemical' classes of the 20 common amino acids have been defined by the physicochemical properties of their side chains. The distinction between hydropathy, volume, chemical, charge, hydrogen donor or acceptor atoms and polarity properties of the side chains was taking into account. "Very similar, similar, dissimilar or very dissimilar" terms were used to qualify the importance of physicochemical properties modifications due to AA changes.

2.3. Location of catalytic amino acids of antibodies displaying amidase activity

Among the 23 antibodies displaying an amidase activity, 15 had been studied by authors at the molecular level in order to identify or hypothesize the AA implicated in the active sites (in bold in Table 4). Most of the active sites were deduced from molecular modeling by sequence homology, except in the case of 43c9, Yvo (crystallographic data, Protein Data Bank accession codes 43c9 and 2agj, respectively) and of 9G4H9 (confirmed by mutagenesis). When authors proposed several hypotheses implying catalytic triads or dyads, we arbitrarily hold the hypothesis of a catalytic triad. When several catalytic triads were proposed, we retained each hypothesis.

2.4. Statistical analysis

Statistical significance was calculated using the following statistical test, with null hypothesis H_0: $p_1 = p_2$ against H_1: $p_1 \neq p_2$

$$Z^{\#} = \frac{p_1 - p_2}{\sqrt{p(1-p)((1/n)+(1/m))}}$$

This statistic, under H_0, is asymptotically normal and the critical region of rejection of H_0 is: $(-\infty, -z_{1-\alpha/2}]$ U $[z_{1-\alpha/2}, +\infty)$, where $(1-\alpha)$ 100% is the confidence level of test and z_α is the quantile of the standard normal distribution $N(0,1)$.

For instance, for $\alpha = 0.05$, $z_{1-\alpha/2}$ corresponds to $z_{0.975} = 1.96$:

$$p_1 = \frac{X}{n}, \quad p_2 = \frac{Y}{m}, \quad p = \frac{X+Y}{n+m}$$

where X is the number of cases in the first sample and Y is the number of cases in the second sample.

This test has been developed for comparing two distinct populations in terms of proportionality (Hoel et al., 1971).

Table 1
Source and characteristics of the studied catalytic antibodies.

Name	Format and class or subclass	Production ways (and references)	Activity
Anti-VIP	LC derived from IgG	BALB/c mice immunization with VIP (Gao et al., 1994)	
DM506 DM408	scFv, *Na*	Selection on phage library from autoimmune human patients (Bangale et al., 2003)	
ECL2B	LC derived from IgG1	BALB/c mice immunization with CCR5 receptor (Mitsuda et al., 2004)	
i41-7	IgG1	BALB/c mice immunization with urease *H. pylori* (Uda and Hifumi, 2004)	
HpU-18-L	LC derived from IgG1		
HpU-9-L	LC derived from IgG1	BALB/c mice immunization with urease *H. pylori* (Hifumi et al., 2005)	
HpU-2-H	HC derived from IgG1	BALB/c mice immunization with urease *H. pylori* (Hifumi et al., 2006)	
Yvo	Fab derived from IgM	Isolated from patients with Waldenström's macroglobulinemia (Ramsland et al., 2006)	
$VL_{2'}$-t 2E6 VLt'-5D3	VL heterodimer, *Na* VL monomer, *Na*	Selection on phage library from human origin (Taguchi et al., 2008)	
UA15-L	LC derived from IgG1	BALB/c mice immunization with recombinant urease (Hifumi et al., 2008)	Amidase
scFv E6 scFv E11	scFv derived from IgG	Reactive immunization of autoimmune prone mice (Durova et al., 2009)	
hk14	*Na*	Selection on phage library from asthmatic human patients (Paul et al., 2001)	
c23.5	LC derived from IgG	BALB/c mice immunization with VIP-KLH conjugate (Paul et al., 2001)	
6B8E12	IgG	Anti idiotypic antibody from immunized BALB/c mice (Pillet et al., 2002)	
41S-2-L	LC derived from IgG2b	BALB/c mouse immunization with a peptide derived from gp41 (Hifumi et al., 2006)	
9G4H9	IgG2b	Anti idiotypic antibody from immunized Biozzi mice (Avalle et al., 1998)	
FT6 FT12	scFv, *Na*	Selection on phage library from BALB/c mouse origin (Tanaka et al., 1999)	
43c9	*Na*	Immunization of mice using TSA (Thayer et al., 1999)	
312D6	IgG2a	Immunization of BALB/c mice using TSA (Aggarwal et al., 2003)	
17E8	IgG2b	Immunization of mice using TSA (Zhou et al., 1994)	
CNJ206	IgG2a	Immunization of BALB/c mice using TSA (Charbonnier et al., 1995)	
48G7	IgG	Immunization of BALB/c mice using TSA (Patten et al., 1996)	
7C8	scFv derived from IgG1	Immunization of BALB/c mice using TSA (Gigant et al., 1999)	
D2.3	IgG2a	Immunization of BALB/c mice using TSA (Gigant et al., 1998; D'Souza et al., 2002)	Esterase
9A8	Fab derived from IgM	Anti idiotypic antibody from immunized Biozzi mice (Kolesnikov et al., 2000)	
3F1	scFv, *Na*	Selection on phage library from immunized mice with TSA (McKenzie et al., 2007)	
6D9	IgG1	Immunization of BALB/c mice using TSA (Oda et al., 2007)	
H11	*Na*	Immunization of BALB/c mice (Brooks et al., 1996)	
39A11	IgG	Immunization of mice using TSA (Romesberg et al., 1998)	
1E9	IgG1	Immunization of mice using TSA (Xu et al., 1999)	Diels Alder
9D9	IgG2a	Immunization of mice using TSA (Hugot et al., 2002; Zheng et al., 2004)	
13G5	IgG1	Immunization of mice using TSA (Zhu et al., 2004; Debler et al., 2009)	
3D8	IgG	Immunization of autoimmune prone mice (Kim et al., 2006)	DNAse
Ig 521	IgG	BALB/c mouse immunization with Cucumber mosaic virus (Zein et al., 2010a)	
21D8	IgG1	Immunization of 129 GIX^+ mice using TSA (Hotta et al., 2000)	Decarboxylase
15A9	IgG1	Immunization of mice using TSA (Golinelli-Pimpaneau et al., 2006)	Transaminase

The data in grey and white boxes indicate the antibodies from murine and human origin, respectively. TSA: transition state analogue; LC: light chain; HC: heavy chain; scFv: single chain fragment variable; Na: isotype is not available. Nature of various tested substrates is described in corresponding publications.

3. Results

3.1. Deviation of catalytic antibody sequences and their germline counterparts

It is commonly admitted that the maturation of antibodies is the result of the selective expansion of B cell clones leading to the expression of high affinity antibodies. The maturation process of the immune system undoubtedly involves hypermutations. The mutation rate of antibodies has been shown to be intimately linked to the measured affinity constants that characterize antibody–antigen interactions, and improves significantly the strength of the affinity (Poulsen et al., 2007). Conversely, polyreactive antibodies that are found in healthy individuals without deliberate immunization or in the absence of obvious advert disease, generally display low specific affinities to a wide variety of self and/or foreign antigens. It is noteworthy that sequences of polyreactive antibodies are near to their germline counterpart (Casali and Notkins, 1989; Zhou et al., 2007). Interestingly, polyreactivity is one of the striking features of many catalytic antibodies (Kalaga et al., 1995; Tawfik et al., 1995; Planque et al., 2004). It is thus conceivable that the link between the polyreactivity of antibodies and their catalytic capacity may find its origin in the extent of somatic hypermutations. To investigate this link, we collected the V domain sequences of 23 catalytic antibodies endowed with amidase activity (Table 2).

Using IMGT/V-QUEST and IMGT/DomainGapAlign as described in Section 2, we individually analyzed VH and VL sequences. Data are gathered in Table 3. Percentages of nucleotide identity suggest that, for catalytic antibodies endowed with amidase activity, the V-domains display a high degree of conservation with their germline counterparts, around $97.5 \pm 1.7\%$ for VL and around $97.0 \pm 2.7\%$ for VH. Our results are in concordance with that of previously obtained on DNAse (Zein et al., 2010a) (99% for VL and 95% for VH) and esterolytic activities (Sun et al., 2001) (98% for VL and 93% for VH).

Considering the non-silent mutations, we have calculated an average of 6.1 and 9.7 AA changes for VL and VH, respectively, yielding an average of 16 AA changes for the VL/VH pairing (Table 4). Such a rate of AA change is lower when compared to the rate that characterizes mature high affinity antibodies. For example, an antibody displaying affinity in the nanomolar range has evolved from the germline by acquiring 13 (for VL) and 14 (for VH) AA changes (Poulsen et al., 2007), yielding 27 AA changes for the VL/VH pairing of this particular antibody. When extending their study to several antibodies, the same authors estimated an average number of 21 AA changes from data concerning VL/VH pairing, i.e. more than 31% higher than the average number of AA changes observed in amidolytic antibodies.

Interestingly, using the IMGT/DomainGapAlign tool, we have estimated the importance of the modifications of the physicochemical properties linked to the AA changes. Despite the low rate of AA

Table 2
Identification indexes of the studied catalytic antibodies.

Name	References	Accession numbers	Activity	
Anti-VIP	Gao et al. (1994)	L34775 (LC)		
DM506	Bangale et al. (2003)	AF509587 (scFv)		
DM408		AF509586 (scFv)		
ECL2B	Mitsuda et al. (2004)	*Nd*		
i41-7	Uda and Hifumi (2004)	*Personal communication*		
HpU-18-L		AB117100 (VL) & AB117101 (VH)		
HpU-9-L	Hifumi et al. (2005)	*Personal communication*	Peptidase	
HpU-2-H	Hifumi et al. (2006)	AB117095 (HC)		
Yvo	Ramsland et al. (2006)	2agj (Fab from IgM)		
$VL_{2'}$-t 2E6	Taguchi et al. (2008)	FJ231715 (VL heterodimer)		
VLt'-5D3		FJ231714 (VL monomer)		
UA15-L	Hifumi et al. (2008)	AB286872 (VL) & AB286873 (VH)		
scFv E6	Durova et al. (2009)	FJ159155 (VL) & FJ159154 (VH)		Amidase
scFv E11		FJ164066 (VL) & FJ164065 (VH)		
hk14	Paul et al. (2001)	1850134 (LC)		
c23.5		896288 (LC)	Protease	
6B8E12	Pillet et al. (2002)	EF392666 (VL) & EF392665 (VH)		
41S-2-L	Hifumi et al. (2002)	D85104 (LC)		
9G4H9	Avalle et al. (1998)	AJ277812 (VL) & AJ277813 (VH)		
FT6	Tanaka et al. (1999)	*Nd*	β-Lactamase	
FT12		*Nd*		
43c9	Thayer et al. (1999)	43c9		
312D6	Aggarwal et al. (2003)	AY349608 (VL) & AY351681 (VH)	Amidase	
17E8	Zhou et al. (1994)	1eap		
CNJ206	Charbonnier et al. (1995)	1kno		
48G7	Patten et al. (1996)	1aj7		
7C8	Gigant et al. (1999)	1ct8	Esterase	
D2.3	Gigant et al. (1998) and D'Souza et al. (2002)	1kn2		
9A8	Kolesnikov et al. (2000)	AF253060 (VL) & AF253061 (VH)		
3F1	McKenzie et al. (2007)	DQ849033 (scFv)		
6D9	Oda et al. (2007)	2dtm		
H11	Brooks et al. (1996)	L41689 (VL) & L41728 (VH)		
39A11	Romesberg et al. (1998)	1a4k		
1E9	Xu et al. (1999)	1c1e	Diels Alder	
9D9	Hugot et al. (2002) and Zheng et al. (2004)	1lo4		
13G5	Zhu et al. (2004) and Debler et al. (2009)	1ruq		
Ig 521	Zein et al. (2010a)	EF672206 (VL) & EF672220 (VH)	DNAse	
3D8	Kim et al. (2006)	AAF79129 (VL) & AAF79128 (VH)		
21D8	Hotta et al. (2000)	1c5b	Decarboxylase	
15A9	Golinelli-Pimpaneau et al. (2006)	1wc7	Transaminase	

scFv: single chain variable fragment; LC: light chain; HC: heavy chain; VL: variable domain from light chain; VH: variable domain from heavy chain; Nd: sequence not deposited in database, but available, in the publication.

changes, most of the modifications in the physicochemical properties of the amino acids changes of VL and VH domains (64.5% and 65.1%, respectively) were dissimilar or very dissimilar (Table 4).

As described in Section 2, 15 of the 23 catalytic antibodies displaying an amidase activity were studied by the respective authors in order to hypothesize their active site (Table 4, in bold). We have observed that 11 among the 15 proposed active sites were exclusively located on the light chain. Active sites of hk14, c23.5 and HpU-2-H were not hypothesized by authors. However, the hk14 and c23.5 antibody fragments are known to be constituted only by a light chain (Paul et al., 2001), consequently, their active site is *de facto* located on them. Conversely, active site of HpU-2-H is *de facto* located on the heavy chain. Hence, the ratio of active sites exclusively located on light chain becomes 13/18, i.e. around 72%.

Then we have investigated whether the AA implied in the putative catalytic sites originate from the introduced somatic mutations, or are already present in the V genes in germline configuration. In a half of the cases, at least one amino acid in the catalytic triad has been gained upon somatic hypermutation. Among these catalytic amino acids issued from somatic mutations, around 71% concern dissimilar or very dissimilar AA changes (Fig. 1). Hence, the nature of these AA seems to be important for the acquisition of the catalytic activity. Moreover, the data in Fig. 1 shows that location of catalytic Amino acids is not necessarily focused on Complementary Determining Region (CDR) (notably on the VH CDR3) as one might expect for standard antigen binding. On the contrary, catalytic amino acids were also noticed in the framework regions. This suggests that hypermutation mechanisms for acquisition of catalytic function could be different from mechanisms for the improvement of affinity.

3.2. Gene subgroups encoding catalytic antibodies

As previously mentioned, in most of the cases, the putative catalytic site involves amino acid located on the light chains of catalytic antibodies. Thus we further focused on the genes encoding light chains of catalytic antibodies. To this end, the frequencies of the IGKV germline genes used by catalytic antibodies (Table 3) were compared with that reported in IMGT/GeneFrequency (Lefranc et al., 2009).

There are 1553 IGKV cDNA sequences expressing IGKV of *Mus musculus* origin from IMGT/LIGM-DB listed in the IMGT/GeneFrequency tool. Reported IGKV genes have not been observed with identical frequencies. We have considered that gene subgroups are rarely represented when their frequency of appearance in the database is lower than 5%. Rare gene subgroups (IGKV

Table 3
Germline origin and percentage of nucleotidic sequence identity of catalytic antibodies displaying amidase activity.

Name	Species	Light chain				Heavy chain				
		V-GENE	Id (%)	J-GENE	Id (%)	V-GENE	Id (%)	J-GENE	Id (%)	D-GENE
Anti-VIP	Musmus	IGKV1-135*01	98.30	IGKJ1*01	100.0			–		
DM506	Homsap	IGKV4-1*01	94.28	IGKJ1*01	97.37	IGHV4-39*03	97.93	IGHJ4*02	89.58	IGHD3-3*01
DM408	Homsap	IGLV1-40*01	97.57	IGLJ2*01	78.95	IGHV3-23*04	100.0	IGHJ4*02	95.83	IGHD6-19*01
ECL2B	Musmus	IGKV1-117*01	98.98	IGLJ1*01	100.0			–		
i41-7	*Musmus*	*IGKV6-25*01*	*Na*	*IGKJ5*01*	*Na*	*IGHV1S135*01*	*Na*	*IGHJ4*01*	*Na*	*Na*
HpU-18-L	Musmus	IGKV1-117*01	97.28	IGKJ4*01	100			–		
HpU-9-L	*Musmus*	*IGKV1-88*01*	*Na*	*IGKJ2*01*	*Na*			–		
HpU-2-H	Homsap		–			IGHV1-77*01	93.06	IGHJ3*01	97.87	IGHD2-14*01
Yvo	*Homsap*	*IGKV3-20*01*	*Na*	*IGKJ1*01*	*Na*	*IGHV2-5*01*	*Na*	*IGHJ4*01*	*Na*	*Na*
$VL_{2'}$-t 2E6	Homsap	IGLV2-14*01	98.61	IGLJ2*01	86.84			–		
	Homsap	IGKV1-39*01	98.57	IGKJ1*01	91.67					
VLt′-5D3	Homsap	IGKV3-20*01	98.23	IGKJ2*01	100.0			–		
UA15-L	Musmus	IGKV1-135*01	99.66	IGKJ1*01	100.0			–		
scFv E6	Musmus	IGKV9-124*01	99.28	IGKJ1*01	91.89	IGHV1-69*01	95.83	IGHJ2*01	85.37	IGHD3-2*02
scFv E11	Musmus	IGKV19-93*01	97.49	IGKJ2*03	94.87	IGHV1-52*01	95.83	IGHJ2*01	75.61	IGHD2-12*01
hk14	*Homsap*	*IGKV1-39*01*	*Na*	*IGKJ4*01*	*Na*			–		
c23.5	*Musmus*	*IGKV1-135*01*	*Na*	*IGKJ1*01*	*Na*			–		
6B8E12	Musmus	IGKV4-61*01	94.20	IGKJ5*01	96.88	IGHV5-6-3*01	99.31	IGHJ1*01	91.67	IGHD2-14*01
41S-2-L	Musmus	IGKV2-137*01	95.92	IGKJ2*01	100.0			–		
9G4H9	Musmus	IGKV2-137*01	96.26	IGKJ5*01	94.44	IGHV1-39*01	100.0	IGHJ2*01	95.74	IGHD2-14*01
FT6	*Musmus*	*IGKV4-55*01*	*Na*	*IGKJ2*03*	*Na*	*IGHV5-6*03*	*Na*	*IGHJ3*01*	*Na*	*Na*
FT12	*Musmus*	*IGKV4-55*01*	*Na*	*IGKJ5*01*	*Na*	*IGHV5-9-3*01*	*Na*	*IGHJ3*01*	*Na*	*Na*
43c9	*Musmus*	*IGKV8-24*01*	*Na*	*IGKJ1*01*	*Na*	*IGHV2-6-4*01*	*Na*	*IGHJ3*01*	*Na*	*Na*
312D6	Musmus	IGKV3-2*01	97.59	IGKJ1*01	94.12	IGHV1-82*01	94.04	IGHJ2*01	91.49	IGHD1-2*01

Data in italic were deduced using the IMGT/DomainGapAlign tool, whereas others were deduced using IMGT/V-QUEST. Id (%) indicates the percentage of identity in nucleotide sequences. When germline identification is deduced by IMGT/DomainGapAlign tool, this percentage is not available (Na) because of the nucleotide sequence is not published. (–) depicts antibody fragment that lacks the indicated chain. The data in grey and white boxes indicate the antibodies from murine and human origin, respectively.

2, 5, 7, 9, 10, 11, 13, 15, 16, 17 and 19) thus represent 251 sequences among the 1553 in IMGT/GeneFrequency, i.e. 16.1% of all sequences.

In the present analysis, 16 murine IGKV genes were identified among the 23 catalytic antibody V-KAPPA sequences (Table 3, grey boxes). Unexpectedly, some of the rare gene subgroups (IGKV2, IGKV9 and IGKV19) were over-represented (about 4/16, i.e. 25%) among catalytic antibodies. In particular, the frequency ratios calculated in the case of IGKV2 and IGKV19 were significantly different

Table 4
Location of catalytic amino acids and somatic mutation rates of catalytic antibodies displaying amidase activity.

Name	Location of catalytic amino acids	Light chain	Heavy chain
		dis&vdis AA/total AA changes	dis&vdis AA/total AA changes
Anti-VIP	VL	3/4	–
DM506	VL	7/13	4/5
DM408	VL	4/6	3/3
ECL2B	VL	2/2	–
i41-7	VL or VH	2/2	5/9
HpU-18-L	VL	2/8	–
HpU-9-L	VL	2/4	–
HpU-2-H[a]	VH[a]	–	9/13
Yvo	VL and VH	6/11	10/18
$VL_{2'}$-t 2E6	VL	5/5	–
		2/3	
VLt′-5D3	VL	1/4	–
UA15-L	VL	1/1	–
scFv E6	Nd	2/2	8/11
scFv E11	Nd	4/4	8/12
hk14[#]	VL[#]	10/14	–
c23.5[#]	VL[#]	3/5	–
6B8E12	Nd	11/14	3/3
41S-2-L	VL	6/7	–
9G4H9	VL	5/7	0/0
FT6	Nd	5/8	10/16
FT12	Nd	2/6	7/13
43c9	VL and VH	4/6	9/13
312D6	VL and VH	2/5	6/10
23 antibodies	11 exclusively on VL	91/141 = 64.5% dis&vdis AA	82/126 = 65.1% dis&vdis AA
15 studied active sites	2 *de facto* on VL (#)	141/23 = 6.1 AA changes	126/13 = 9.7 AA changes
3 deduced *de facto* (#)	1 *de facto* on VH (#)		

The data in grey and white boxes indicate the antibodies from murine and human origin, respectively. In bold indicates that active sites of corresponding antibodies were identified or hypothesized by authors. For the location of catalytic amino acids, "or" indicates that several hypothesis were proposed, "and" indicates that amino acids located on both chains were suspected. Nd: Not determined active site.

[#] Catalytic amino acids were neither identified nor hypothesized, but the active site is necessarily located on the unique chain of the catalytic antibody fragment. dis&vdis AA/total AA changes indicates the number of dissimilar or very dissimilar AA changes in strands and loops (IMGT/DomainGapAlign) out of total amino acid changes.

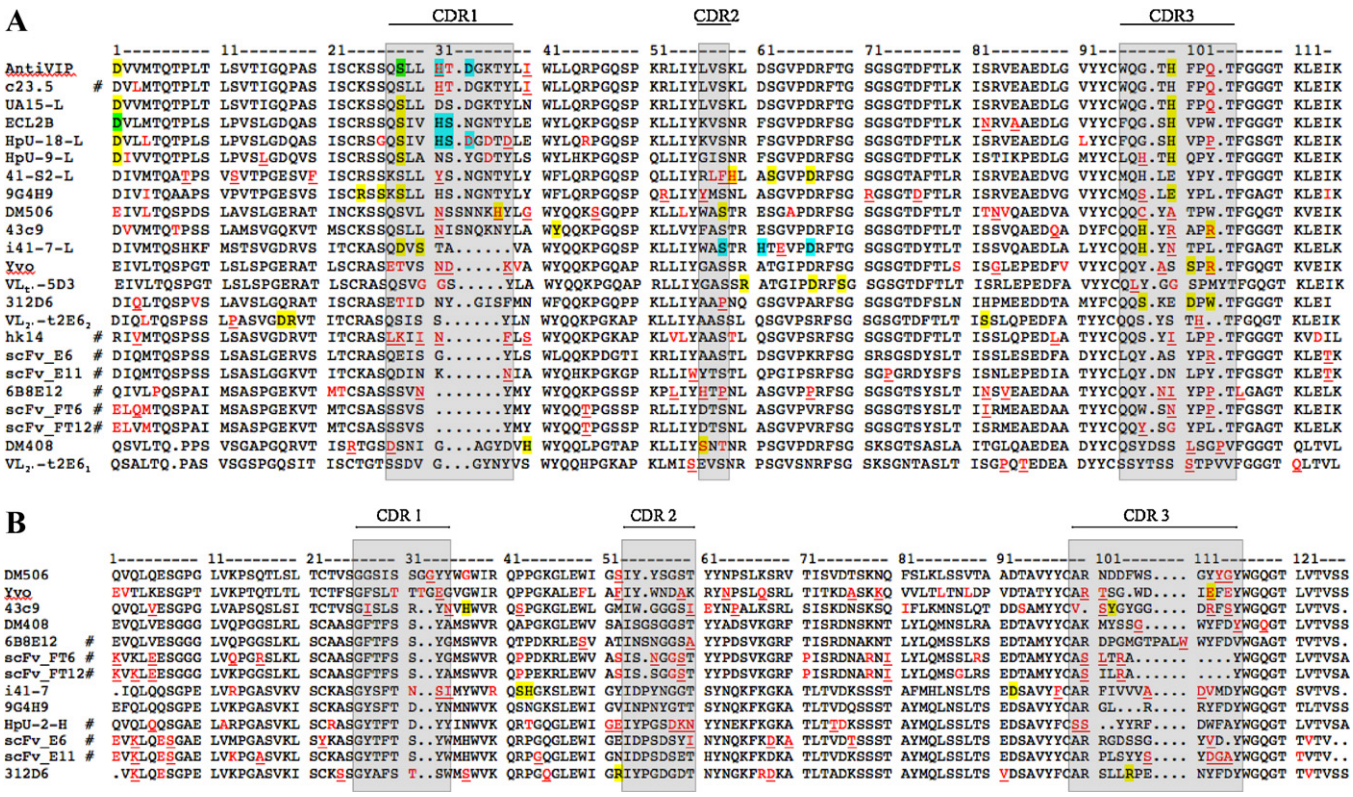

Fig. 1. AA changes and catalytic AA in both chains of catalytic antibodies displaying an amidase activity. Amino acid sequences alignment of VL domains (A) and VH domains (B) were performed using Multalin. The delimitations between the FR and CDR (in grey boxes) have been defined according to IMGT unique numbering (Lefranc et al., 2003; Lefranc, 2011). # indicates that the catalytic AA were neither identified nor hypothesized by authors. In the case of $VL_{2'}$-t 2E6, because this antibody fragment is a VL heterodimer, the first light chain is named $VL_{2'}$-t $2E6_1$, and the second one is named $VL_{2'}$-t $2E6_2$. Here, the active site has been hypothesized to be located on the $VL_{2'}$-t $2E6_2$ monomer. Catalytic AA are defined according to the authors' hypotheses and are highlighted in yellow. When two hypotheses were proposed by the authors, the second one is highlighted in blue. If a AA is suspected to belong to the two potential active sites, it is highlighted in green. Amino acid changes due to somatic mutations are red. Underlined AA are considered as dissimilar or very dissimilar AA changes (according to the IMGT/DomainGapAlign criteria). For example, amino acid H30 from AntiVIP is supposed to be implicated in the active site, results from a somatic hypermutation, and this mutation implies a dissimilar or very dissimilar AA change. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

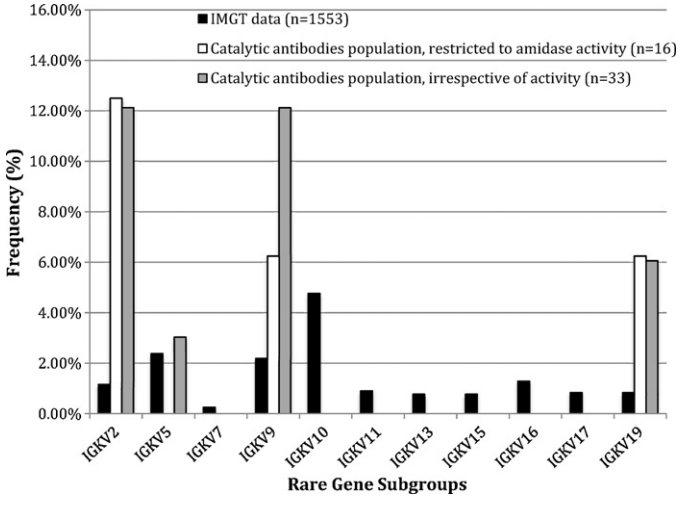

Fig. 2. Comparison of murine IGKV rare gene subgroups frequency. Rare IGKV groups were arbitrarily designed as having a frequency lower than 5%. The "IMGT data" gather 1553 IGKV rearranged sequences from murine origin together, irrespective of their antigen specificity. The "catalytic antibody population, restricted to amidase activity" concerns the 16 mouse Vκ sequences displaying an amidase activity and listed in Table 3 (grey boxes). The "catalytic antibody population, irrespective of activity" concerns the 33 mouse Vκ sequences displaying catalytic activity (the 16 previous sequences + the 17 ones referred in Table 4).

($p < 0.01$ and $p < 0.05$, respectively) when calculated among the population of catalytic antibodies and in IMGT/GeneFrequency (Fig. 2). The small sample size – that could be considered as a possible source of error – is checked and validated by the statistical analysis. It suggests that rare subgroups such as IGKV2 and IGKV19 are preferentially selected for the catalytic expression.

The nature of the catalytic function may not have a particular relevance for the study of gene frequency. Further, the analysis was extended to the catalytic antibodies endowed with other activities, like DNAse, esterase, decarboxylase, transaminase and Diels-Alderase (Table 1). Altogether, 33 murine catalytic antibodies (16 murine V-KAPPA with amidase activity and the 17 murine V-KAPPA with other activities) were included in the analysis. Under such conditions, rare IGKV gene subgroups were used by 33.3% of the catalytic light chains versus 16.1% for the overall antibody population ($p < 0.01$) (Fig. 2). This trend was confirmed when extending the analysis to the study of human V-KAPPA.

Among the 3664 cDNA sequences (1553 murine and 2111 human origin) listed in the IMGT/GeneFrequency tool, 255, i.e. 6.9% sequences (251 murine and 4 human origin) are found to derive from rare gene subgroups. Significantly, 11 sequences (28.9%, $p < 0.001$) have been observed to derive from rare gene subgroups among the population of 38 catalytic antibodies, i.e. 33 murine V-KAPPA sequences (Table 1, grey boxes) and 5 human V-KAPPA sequences (Table 3, white boxes). The present data thus confirm that rarely represented IGKV gene subgroups are more frequently associated with the expression of antibody V-KAPPA domains with catalytic activity.

4. Discussion

Several papers published to date deal with V gene rearrangements of catalytic antibodies produced by following a unique production pathways (Zein et al., 2010a; Sun et al., 2001). This may introduce a bias in the expression profile of V genes. We therefore, compare the expression features of reported catalytic antibodies regardless of their mode of elicitation, namely, deliberate immunization with a TSA, idiotypic pathway and selection on phage library from patients or disease animal models (Table 1). As shown in Table 1, a large majority of the listed catalytic antibodies has undergone class switching into IgG, indicating that the maturation process of the immune response has been activated. This process is further analyzed by investigating the rate of somatic mutations. For this purpose, we identified a population of 23 catalytic antibodies endowed with peer activities (protease, amidase and peptidase). We highlighted a high percentage of identity between the nucleotide sequences of these 23 antibodies and their closest germline V genes, indicating a weak rate of somatic mutation and a low rate of AA change. Nevertheless, mutations were observed to induce important modifications in physicochemical properties of a large majority of the AA changes. Thus, the AA change rate is low in terms of quantity, but seems to be important in terms of quality. Among the 23 amidolytic antibodies, 15 active sites were hypothesized, whereas the active sites of the 8 others have been neither characterized nor hypothesized. In spite of the localization of most of the active sites on the light chain (i.e. 72%), a higher level of AA changes was observed on the VH domain. This may be in order to maintain the specific recognition of the substrate.

Erhan and Greller (1974) have previously suggested that all antibody light chains may have protease-like features. Uda and Hifumi (2004) have further investigated the relationships between the structure of the catalytic triad Asp-Ser-His and the IGKV germline by referring to 49 randomly extracted clones from the Protein Data Bank (PDB) and to 35 clones from their laboratory. They have demonstrated that most of the prospected catalytic triads of these clones were encoded by the corresponding germline amino acids. In the present report, the results suggest that 50% of the hypothesized catalytic triads possess at least one amino acid derived from the process of hypermutation.

Otherwise, the catalytic activities of matured antibodies and their germline counterparts have been compared in various reports (Patten et al., 1996; Ulrich et al., 1997; Gololobov et al., 1999; Romesberg et al., 1998; Xu et al., 1999). The contradictory results led to suggest that a systematic rule cannot be applied generally. Indeed Ulrich et al. (1997) and Gololobov et al. (1999) demonstrated that the antibodies encoded by germline genes were endowed with an equal or greater activity than the matured ones. In contrast, Romesberg et al. (1998) demonstrated that the matured antibody endowed a greater activity than its germline counterpart. Indeed, they have shown that the catalytic antibody 39A11 that catalyzes a Diels Alder reaction is weakly mutated, but a single somatic mutation on the light chain appear to be largely responsible for the catalytic activity. Interestingly, this single mutation (Ser91Val, i.e. Ser107Val according to the IMGT numbering) is considered as a very dissimilar AA change according to the IMGT criteria. Xu et al. (1999) also suggested that subtle mutational steps were able to influence catalytic efficiency. In the same way, Patten and collaborators suggested that the activity of the germline antibody was lower than the matured one. However, these authors have observed that none of the AA changes was implicated in the active site. On the contrary, these AA changes were involved in the conformational structure of the antibodies (Patten et al., 1996).

Finally, the subtle equilibrium between a weak mutation rate but nonetheless leading to modifications of the physicochemical properties should also be considered in light of the following observations: (i) catalytic antibodies are mainly polyreactive (Kalaga et al., 1995; Tawfik et al., 1995; Planque et al., 2004), (ii) polyreactive antibodies (but not catalytic) are known to be derived from slightly mutated or unmutated germline V genes (Zhou et al., 2007), and (iii) a decline in the occurrence of catalytic antibodies was observed when repeated versus short immunization protocols were performed (Tawfik et al., 1995). This latter point suggests that the maturation process have to be moderated for the acquisition of the catalytic function. All together, these observations tend toward the same conclusion, i.e. the somatic mutations characterizing catalytic antibodies are quantitatively rare but could be qualitatively significant.

It is commonly accepted that high affinities are related to the somatic mutations appearing during the course of the immune response. Because the catalysis mechanism involves both regeneration of catalysts and product release, abzymes have to show some flexibility properties, and thus must not display high affinities towards antigens. Herein, we showed that the genes encoding catalytic antibodies and the CDR in particular are globally weakly mutated. The catalytic proficiency may be probably more linked to the overall plasticity of weakly mutated V regions versus those of maturated ones. Indeed, somatic mutations generally reduce the conformational freedom of CDR, thus conferring higher affinities (Patten et al., 1996; Wedemayer et al., 1997; Venkateshrao et al., 2004). Conversely, a weak rate of mutations may maintain the possibility of adopting many conformations by CDR which appears to be more compatible with the catalysis mechanisms (i.e. substrate recognition, catalyst regeneration and product release) (Ma and Nussinov, 2010).

The gene subgroups encoding catalytic antibodies were further investigated. Since many sequences of catalytic antibodies are now accumulated in databases, this analysis is from now on statistically relevant. The statistical analysis showed that catalytic antibodies are significantly and preferentially expressed by rare genes subgroups. This observation could find an explanation in a more or less sensitivity of genes to the mutation mechanisms. Indeed, Berek and Milstein (1987) suggested that all V genes might not be equally subjected to somatic hypermutation process. Since then, the somatic hypermutation processes were investigated and various results suggest that mechanisms involved notably depend on the sequence of DNA (Wang et al., 2010; Ganesh and Neuberger, 2011). This could explain the observations of Berek and Milstein suggesting that some V genes are more or less sensitive to hypermutation process. The fact that rare gene subgroups are overexpressed by catalytic antibodies may reflect a different susceptibility or accessibility of certain genes to mutation processes. It would suggest that more accessible genes being more “mutable” and less accessible genes being less mutable. The immune system is intended to produce high affinity antibodies. Consequently, the genes that are more sensitive to hypermutation process would be expressed more frequently. On the contrary, genes unfavorable to hypermutation process would be rarely expressed. On that account, a catalytic repertoire not requiring high level of mutations could more frequently express the “less sensitive” genes. This hypothesis is supported by the results in the present paper.

Finally, the idea that some rare events might be activated in a particular immunological context is innovative, but results from convergence of observations. Indeed, R. Lerner recently proposed that an “S.O.S component” of the human immunological repertoire could explain the activation of rare events leading to the over representation of a specific set of genes (Lerner, 2011). Library approaches seems favor such observations (Smirnov et al., 2011). Whatever the reason, i.e. an emergency situation leading to an unusual accommodation of the immune system, or an autoimmune pathology inducing a deregulation of the immune response, a new

concept emerges and may lays the foundations of a new field of the Immunology.

This study illustrates the complexity of the immune system. It underlines the characteristics of the various repertoires of expressed V genes: mutated in high affinity antibodies versus unmutated in low-affinity antibodies (with polyreactive or catalytic properties). A tendency emerges, showing a higher expression frequency of rare subgroup genes for catalytic antibodies. In this context, the special case of the high occurrence of catalytic antibodies in autoimmunes diseases (Shuster et al., 1992; Ponomarenko et al., 2002, 2006; Wootla et al., 2008, 2011) could be explained by a deregulation of the immune response that leads to the favorable expression of usually rare expressed gene subgroups.

In conclusion, we showed that catalytic antibodies displayed a low rate of somatic mutations. A significant modification in physicochemical properties due to AA changes of mutated was observed. Remarkably, we noticed that the catalytic antibodies are more frequently expressed by rare genes subgroups than binding antibodies. The results herein discussed are not only innovative but also essential to understand the origin of genes encoding catalytic antibodies. By highlighting astonishing rare events occurred in immune response, this paper would provide a basis for future studies in the characterization of genes encoding catalytic antibodies.

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Factor VIII-hydrolyzing IgG in acquired and congenital hemophilia

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ABSTRACT

Anti-factor VIII (FVIII) inhibitory IgG may arise as alloantibodies to therapeutic FVIII in patients with congenital hemophilia A, or as autoantibodies to endogenous FVIII in individuals with acquired hemophilia. We have described FVIII-hydrolyzing IgG both in hemophilia A patients with anti-FVIII IgG and in acquired hemophilia patients. Here, we compared the properties of proteolytic auto- and allo-antibodies. Rates of FVIII hydrolysis differed significantly between the two groups of antibodies. Proline-phenylalanine-arginine-methylcoumarinamide was a surrogate substrate for FVIII-hydrolyzing autoantibodies. Our data suggest that populations of proteolytic anti-FVIII IgG in acquired hemophilia patients are different from that of inhibitor-positive hemophilia A patients.

1. Introduction

Acquired hemophilia is a rare bleeding disorder, caused by the spontaneous occurrence of autoantibodies directed against endogenous factor VIII (FVIII) that inhibit the procoagulant activity of FVIII and are referred to as FVIII inhibitors [1]. About 50% of the cases are associated with underlying pathological conditions. The remaining cases arise during the post-partum period or are idiopathic. FVIII inhibitors also arise in patients with congenital hemophilia A as alloantibodies directed to therapeutically administered exogenous FVIII in the course of replacement therapy [2]. Autoantibodies and alloantibodies against FVIII share similar properties [3,4], but may differ in their kinetics of FVIII inactivation [5].

We have reported that IgG from hemophilia A patients and from patients with acquired hemophilia both may hydrolyze FVIII [6,7]. In the case of hemophilia A, FVIII-hydrolyzing IgG were detected in 50% of inhibitor-positive patients [8]. FVIII-hydrolyzing IgG from these patients inactivated FVIII with kinetics of FVIII degradation that are compatible with a pathogenic role towards therapeutical FVIII [9]. The rates of IgG-mediated FVIII hydrolysis correlated with the inhibitory activity measured in patients' plasma. Cleavage sites for IgG on FVIII were spread over the heavy and light chains of FVIII. In the case of acquired hemophilia, autoimmune FVIII-hydrolyzing IgG were found in 47% of the patients [7]. We failed to find a correlation between the rates of IgG-mediated FVIII hydrolysis and the inhibitory titer in plasma or the survival of the patients one year following diagnosis of the disease. The levels of IgG-mediated FVIII hydrolysis co-evolved with the inhibitory titers measured during the course of the treatment [7].

Here, we decipher the properties of auto-immune FVIII-hydrolyzing IgG and compare them to that of alloimmune FVIII-hydrolyzing IgG. The data suggest that populations of FVIII-hydrolyzing

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IgG are different according to the allo- or auto- immune pathological situation that prevails when they are generated.

2. Materials and methods

2.1. Patients

Plasma samples from 45 patients with acquired hemophilia were obtained from CHU de Rouen (Etude Sacha), Caen, Compiègne, from Hôpital Cochin (Paris), Hôpitaux du Kremlin-Bicêtre (Bicêtre) and Rennes (France), in accordance with the local ethical regulation. Patients' clinical characteristics included in the cohort have been published [7]. Plasma was obtained at the time of diagnosis. Age at time of diagnosis was 68.3 ± 17.6 years (mean ± S.D., ranging from 25 to 92). Patients included 28 males and 13 females (not documented in the case of 4 patients). Inhibitory titer and residual FVIII activity in plasma at time of diagnosis were 84.1 ± 174.0 BU/ml and 5.6 ± 6.2%, respectively. Survival one year after diagnosis, documented for 35 patients, was 60%. In the case of 8 patients, blood samples were obtained sequentially over periods of 6–140 days. For these 8 patients, the underlying treatments included cortico-therapy, therapeutic FVIII, recombinant activated factor VII and/or intravenous immunoglobulins (IVIg).

2.2. IgG purification

IgG was isolated from plasma on protein G Sepharose (Amersham). A therapeutic preparation of pooled normal human immunoglobulin (IVIg, Sandoglobulin®, CSL-Behring, Switzerland) was used as a source of normal IgG. To exclude potentially contaminating proteases, size-exclusion chromatography of patients' IgG and IVIg was performed on a superose-12 column (Amersham) equilibrated with 50 mM Tris, 8 M urea and 0.02% NaN_3 (pH 7.7), at a flow rate of 250 μl/min. IgG was dialyzed against PBS–0.01% NaN_3 for 48 h at 4 °C, followed by dialysis against 50 mM Tris (pH 7.7), 100 mM glycine, 0.02% NaN_3, 5 mM $CaCl_2$ (catalytic buffer) for 24 h at 4 °C.

2.3. Hydrolysis of biotinylated antigens

Commercially available recombinant human FVIII (Kogenate® FS, Bayer HealthCare, France) and activated factor VII (FVIIa, NovoSeven®, Novo Nordisk, Chartres, France) were biotinylated [7]. Biotinylated FVIII and FVIIa (185 nM) were incubated in catalytic buffer with IgG (67 nM) for 24 h at 37 °C. Samples were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Western blotting and detection using streptavidin-coupled alkaline phosphatase (KPL, Gaithersburg, MD, USA) and the BCIP/NBT substrate. The rates of hydrolysis of FVIII were measured as described [7]. Results were expressed as millimoles of FVIII protein hydrolyzed per minute per moles of IgG. Significant differences between the rates of hydrolysis by patients' IgG and by IVIg were assessed using an ANOVA post hoc test (Ficher PLSD). Reported P values are one-sided.

2.4. Pro-Phe-Arg-MCA hydrolysis

IgG (67 nM) was mixed with proline-phenylalanine-arginine-methylcoumarinamide (PFR-MCA, Peptide Inc., Osaka, Japan) at 100 μM in 40 μl catalytic buffer and incubated in the dark for 24 h at 37 °C. Hydrolysis was determined by the fluorescence of the leaving group (aminomethylcoumarin; λ_{em} 465 nm, λ_{ex} 360 nm). Fluorescence values were compared to a standard curve of free MCA to compute corresponding quantities of released MCA. At each time point, background release of MCA, measured for each PFR-MCA concentration in wells containing the substrate alone, was subtracted from the value observed in the presence of the antibodies.

2.5. Calculation of K_i

IgG (67 nM) was incubated with 60, 120 and 180 μM of PFR-MCA in the presence of increasing amount of FVIII (0.013–3.3 μM), for 24 h at 37 °C. For each FVIII concentration, a double reciprocal graph was plotted: the reciprocal of the rate of PFR-MCA hydrolysis versus that of the substrate concentration (PFR-MCA). The slopes of the curves were calculated by linear regression analysis, and plotted as a function of inhibitor concentration (i.e., FVIII). Intersection with the X-axis of the linear regression curve obtained with the latter data, indicates the K_i value.

2.6. Determination of the quantity of catalytic antibodies in plasma

IgG from patients 1, 2, 15, 20, 22, 38, 42, 43, 45 were allowed to react with Pefabloc® (0–0.05 μM) (Roche Diagnostics, Mannheim, Germany), an irreversible inhibitor of serine proteases, for 1 h at 37 °C. Mixtures were incubated with PFR-MCA (100 μM) for 24 h at 37 °C. Residual PFR-MCA hydrolysis was measured. Inhibition data (total inhibition values) obtained were curve-fitted to obtain single linear regression curves that represent IgG with low PFR-MCA hydrolyzing activity (Q_L). Values calculated from the linear regression curve at each Pefabloc® concentration were subtracted from total inhibition values; obtained data were curve-fitted to obtain a second single linear regression curve that represents IgG with high PFR-MCA hydrolyzing activity (Q_H). Theoretically, the amount of inhibition by Pefabloc® is a reflection of the quantity of catalytic sites in the IgG pool. Intersection of each generated regression curve with the X-axis indicates the amounts of IgG with low and high PFR-MCA hydrolyzing activity, respectively.

3. Results

3.1. IgG from patients with acquired hemophilia hydrolyze FVIII

Hydrolytic patterns of FVIII and FVIIa incubated in buffer alone or with purified IgG from five patients are depicted in Fig. 1A. FVIII (185 nM) incubation with IgG (67 nM) from patients 2, 10, 16 and 32 resulted in FVIII hydrolysis. The FVIII migration profile exhibited minimal spontaneous degradation when it was incubated in buffer alone (Ctl). In parallel, trypsin (>0–41.7 nM) incubated with FVIII displayed a different hydrolytic pattern as compared to that of FVIII hydrolysis by IgG (data not shown). IgG from acquired hemophilia patients did not hydrolyze FVIIa or human serum albumin (Fig. 1A and [7]). Specific rates of FVIII hydrolysis by the IgG from 45 acquired hemophilia patients were calculated using the densitometric analysis of the hydrolyzed bands. IVIg exhibited a hydrolytic activity of 0.06 ± 0.03 mmol/min/mol (Table 1 and Fig. 1E). IgG from 21 of the 45 patients (46.6%) exhibited a FVIII-hydrolyzing activity significantly higher than that of IVIg (Table 1, $P < 0.05$). The mean hydrolytic activity of IgG of these 21 patients was 0.22 ± 0.09 mmol/min/mol (coefficient of variation: 0.29 ± 0.20) [7].

3.2. IgG-mediated PFR-MCA hydrolysis correlates with rates of FVIII hydrolysis

An excess of FVIII neutralized in a dose-dependent manner the auto-immune IgG-mediated hydrolysis of PFR-MCA (Fig. 1B). The Ki calculated in the case of IgG from patient 1 was 4.45 ± 0.49 μM. An excess of PFR-MCA inhibited FVIII hydrolysis

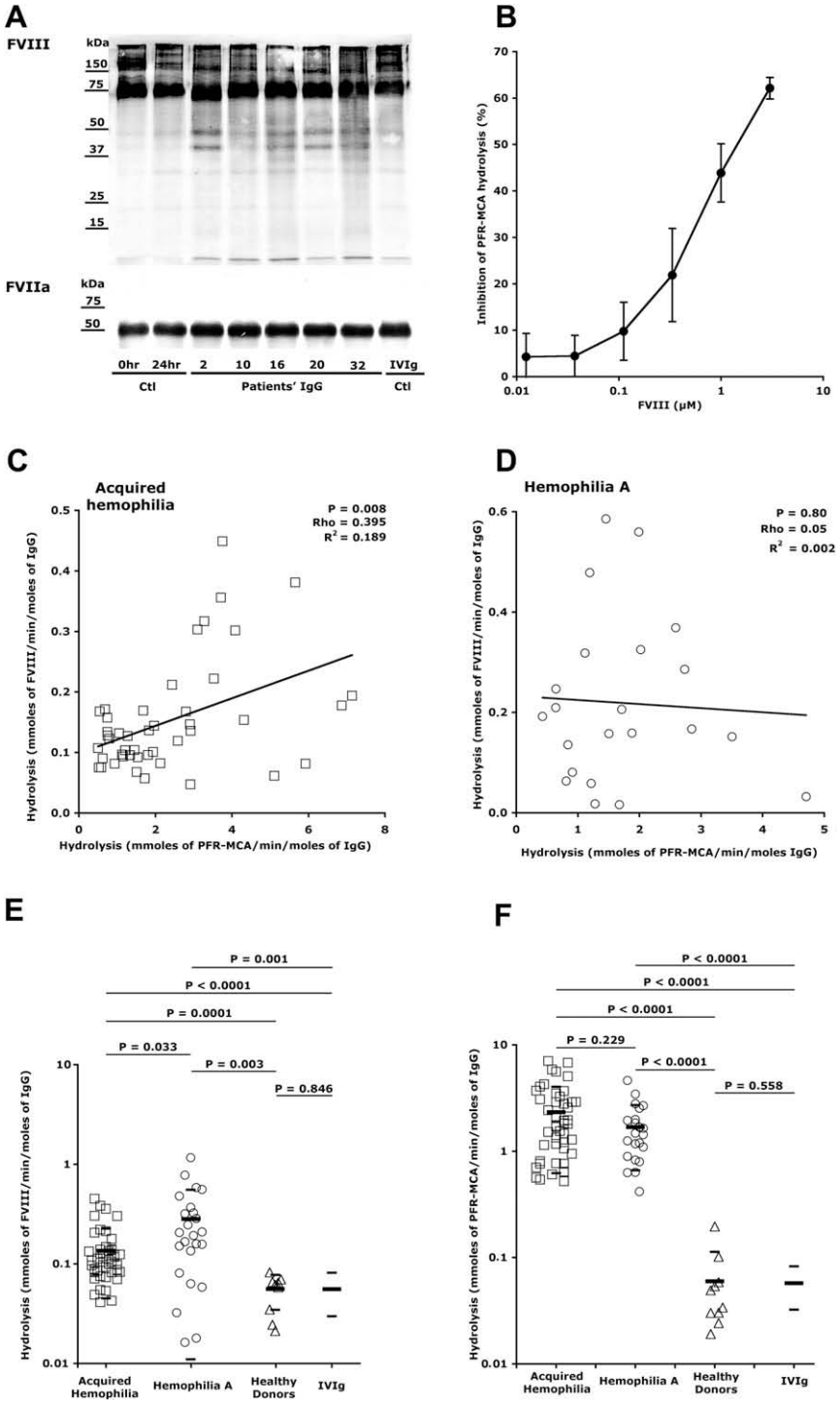

Fig. 1. (Panel A) Hydrolysis of FVIII by IgG purified from the plasma of patients with acquired hemophilia. Biotinylated FVIII and FVIIa (185 nM) were incubated alone (Ctl) or in the presence of IgG (67 nM) from patients 2, 10, 16, 20 and 32 for 24 h at 37 °C. IVIg was used as a source of normal IgG and as a negative control. Migration profiles on a 10% SDS–PAGE are depicted. (Panel B) Inhibition of hydrolysis of PFR-MCA by purified IgG in the presence of FVIII. IgG (67 nM) from patient 1 was allowed to react with increasing concentrations of FVIII (0–3.3 μM) for 30 min at 37 °C. The mixtures were then incubated in the presence of PFR-MCA (60 μM) for 24 h at 37 °C. The figure depicts the inhibition of IgG-mediated PFR-MCA hydrolysis in the presence of FVIII, measured in two independent experiments. The K_i was calculated as described in the materials and methods and was equal to 4.45 ± 0.49 μM. (Panels C and D) Correlation between the hydrolysis of FVIII and PFR-MCA by IgG from patients' IgG. Biotinylated FVIII (185 nM) and the peptide PFR-MCA (100 μM) were incubated alone or in the presence of IVIg or of IgG (67 nM) purified from the plasma of 45 patients with acquired hemophilia (Panel C) or 22 patients with congenital hemophilia A (Panel D, [9]), for 24 h at 37 °C. Rates of hydrolysis of FVIII and of PFR-MCA were calculated as explained in Materials and Methods and are expressed as mmoles of substrate hydrolyzed per min per moles of IgG. Data were plotted for all patients (open squares and circles). In each panel, the solid curve represents the linear regression analysis of the data. The significance of the correlations between the FVIII- and PFR-MCA-hydrolyzing activities were assessed using the nonparametric Spearman rank correlation. (Panels E and F) IgG-mediated hydrolysis of FVIII and of PFR-MCA. Rates of hydrolysis of FVIII (Panel E) and of PFR-MCA (Panel F) were measured in the case of IgG purified from the plasma of 45 patients with acquired hemophilia, 22 patients with congenital hemophilia A and 9 healthy donors (panel E, 10 healthy donors in the case of panel F). Hydrolysis mediated by IVIg in the case of both substrates is indicated. The thick lines represent the arithmetic means and the thin lines the standard deviations. Significance between differences were assessed using the nonparametric Mann–Whitney test.

Table 1
Specific rates of hydrolysis of FVIII and of PFR-MCA by IgG purified from the plasma of 45 patients with acquired hemophilia.

Patient number	Inhibitory titer in plasma[a] (BU/ml)	Hydrolysis of FVIII[b] (mmol/min/mol)	Hydrolysis of PFR-MCA[c] (mmol/min/mol)
1	40	0.45 ± 0.2*	3.78 ± 1.4*
2	63	0.15 ± 0.0	4.34 ± 0.5*
3	128	0.14 ± 0.1	2.00 ± 0.9*
4	114	0.13 ± 0.1	1.31 ± 0.7*
5	380	0.17 ± 0.0	0.57 ± 0.1
6	32	0.12 ± 0.1	0.82 ± 0.6*
7	49.4	0.08 ± 0.0	0.96 ± 0.1*
8	3.1	0.38 ± 0.2*	5.68 ± 1.8*
9	42	0.13 ± 0.0	1.08 ± 0.1*
10	6	0.17 ± 0.0	0.71 ± 0.1*
11	14	0.08 ± 0.0*	0.55 ± 0.1*
12	2	0.12 ± 0.1	2.61 ± 0.8*
13	40	0.16 ± 0.1*	0.78 ± 0.7
14	2	0.22 ± 0.0*	3.56 ± 0.6*
15	80	0.18 ± 0.0*	6.90 ± 0.6*
16	52	0.14 ± 0.0*	1.86 ± 0.2*
17	100	0.36 ± 0.1*	3.74 ± 0.7*
18	10	0.09 ± 0.0	1.57 ± 0.3*
19	1.4	0.10 ± 0.0	1.84 ± 0.1*
20	18	0.08 ± 0.0	5.96 ± 0.9*
21	1050	0.15 ± 0.1*	2.94 ± 0.3*
22	330	0.14 ± 0.1*	2.95 ± 1.1*
23	18	0.30 ± 0.1*	4.12 ± 0.7*
24	1.3	0.05 ± 0.0	2.94 ± 0.5*
25	60	0.06 ± 0.0	5.14 ± 0.2*
26	1	0.06 ± 0.0	1.75 ± 0.8*
27	4	0.10 ± 0.1	1.16 ± 0.3*
28	56	0.07 ± 0.0	1.54 ± 0.1
29	4.5	0.13 ± 0.0*	0.77 ± 0.2*
30	1.5	0.08 ± 0.0	0.62 ± 0.2*
31	3	0.11 ± 0.1*	0.53 ± 0.0
32	7	0.19 ± 0.0*	7.17 ± 0.3*
33	362.7	0.08 ± 0.0	2.16 ± 0.1*
34	249.2	0.09 ± 0.1	1.19 ± 0.3*
35	62	0.32 ± 0.0*	3.31 ± 0.1
36	54	0.21 ± 0.1*	2.47 ± 0.1*
37	1	0.13 ± 0.1*	0.78 ± 0.2*
38	105.4	0.10 ± 0.0	1.24 ± 0.3*
39	92.5	0.09 ± 0.0	0.65 ± ± 0.2
40	26.6	0.10 ± 0.1	1.36 ± 0.2*
41	10	0.17 ± 0.0*	2.83 ± 0.6*
42	29.9	0.10 ± 0.0	1.48 ± 0.1*
43	4.9	0.30 ± 0.2*	3.13 ± 1.3*
44	13	0.10 ± 0.0	1.97 ± 0.1*
45	58.2	0.17 ± 0.0*	1.71 ± 0.3*
IVIg	0	0.06 ± 0.03	0.06 ± 0.03

[a] Inhibitory titers were measured in the plasma using the Bethesda assay.
[b] The results are mean ± S.D. 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 0.37 ± 0.21 (coefficients of variation vary between 0.05 and 0.92).
[c] The results are mean ± S.D. of five independent experiments. Spontaneous hydrolysis that occurred upon incubation of PFR-MCA in the presence of buffer alone was subtracted from each analysis. The mean coefficient of variation was 0.37 ± 0.21 (coefficients of variation vary between 0.05 and 0.92).
* $P < 0.05$ for the comparison with intravenous immunoglobulin (IVIg), using an analysis of variance post hoc test.

by IgG in a dose-dependent manner (data not shown). The rate of PFR-MCA hydrolysis of patients' IgG was 2.4 ± 1.7 mmol/min/mol (Table 1 and Fig. 1F). IgG from 35 patients (77.8%) displayed significantly higher hydrolytic rates of PFR-MCA than IVIg (0.06 ± 0.03 mmol/min/mol, $P < 0.05$). FVIII hydrolysis rates and PFR-MCA hydrolysis rates by IgG from the 45 patients were positively correlated (Fig. 1C, Spearman rank correlation, rho = 0.395, $P = 0.008$), which was not the case for IgG from 22 with hemophilia A patients (Fig. 1D, rho = 0.05, $P = 0.8$, [9]). Taken together, the results suggest that PFR-MCA is a suitable alternative substrate for autoimmune FVIII-hydrolyzing IgG.

3.3. Kinetics of PFR-MCA hydrolysis by catalytic antibodies

Using PFR-MCA as a substrate, we quantified the number of enzyme active sites in the polyclonal pool of IgG from patients with acquired hemophilia. Co-incubation of PFR-MCA with IgG from patient 20 with the protease inhibitors E-64 (28 μM), EDTA (1.3 μM), or pepstatin (10 μM) did not inhibit the hydrolytic activity. Conversely, aprotinin (0.3 μM), leupeptin (100 μM) and Pefabloc® (100 μM) inhibited in a dose-dependent way PFR-MCA hydrolysis, suggesting that IgG possess serine protease-like activity (data not shown). In order to calculate the enzyme active sites in the IgG pool, 5.3 pmoles of IgG from patients 1, 2, 15, 20, 22, 38, 42, 43 and 45 were reacted with Pefabloc® (0–0.05 μM) prior to incubation with PFR-MCA (100 μM) (Fig. 2A). IgG with low and high catalytic activity represented approximately 53 ± 11% and 9 ± 4% of total IgG, respectively (Table 2, calculated as explained in Section 2).

Incubation of patients' IgG (67 nM) with increasing concentrations of PFR-MCA (0–2 mM) led to the saturation of hydrolytic activity towards PFR-MCA (Fig. 2B for IgG from patient 2). Fitting the experimental data to Michaelis–Menten equation allowed derivation of the apparent V_{max} and average K_m of the reactions (Table 3). The computed kinetic parameters were heterogeneous among

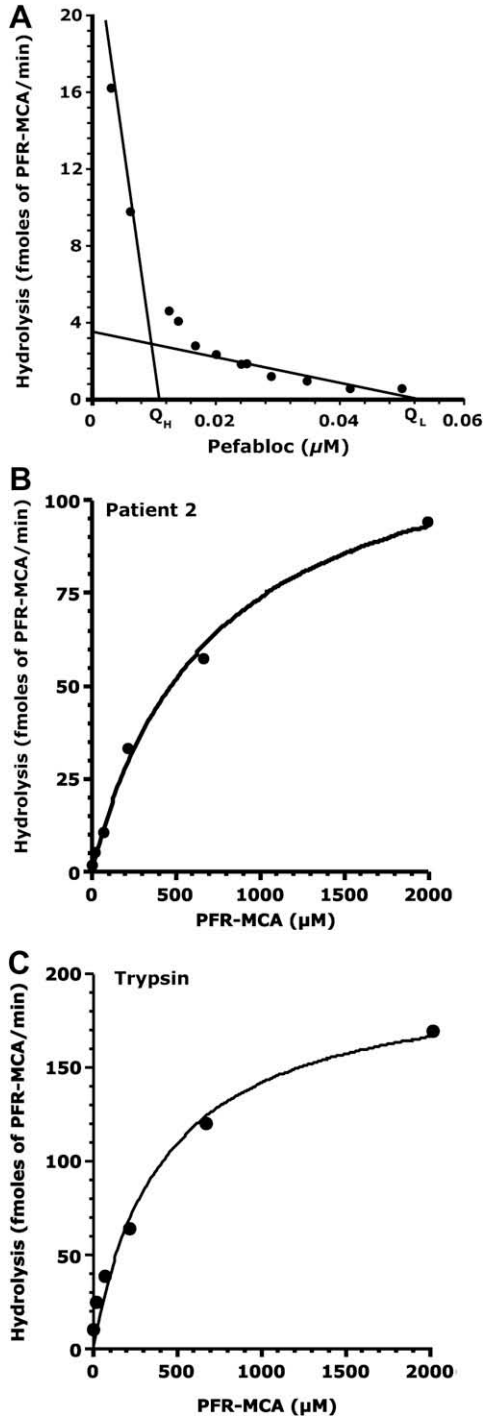

Fig. 2. (Panel A) Quantification of IgG with serine protease activity within the polyclonal pool of IgG from a patient with acquired hemophilia. The IgG purified from the plasma of patient 2 was allowed to react with increasing concentrations of Pefabloc® prior to be incubated in the presence of PFR-MCA. The obtained data were fitted to a double linear regression model to obtain the quantity of catalytic IgG with high PFR-MCA-hydrolyzing activity (Q_H) and the quantity of catalytic IgG with low PFR-MCA-hydrolyzing activity (Q_L),as explained in Section 2. (Panels B and C) Kinetic parameters of PFR-MCA hydrolysis. PFR-MCA (0-2 mM) was incubated in the presence of IgG from patient 2 (Panel B, 66.6 nM) or trypsin (Panel C, 0.42 nM) for 24 h at 37 °C. The plotted values represent the rate of hydrolysis of PFR-MCA determined at each concentration of PFR-MCA. The solid curve depicts the fit of the experimental data to the Michaelis–Menten equation.

patients' IgG, with an apparent V_{max} of 74.7 ± 54.4 fmol/min and an average K_m of 427.4 ± 143.6 μM. Kinetic parameters of PFR-MCA hydrolysis were also calculated for a model serine protease, trypsin, tested at 0.42 nM (Fig. 2C). Vmax and Km for trypsin were 201.4 ± 3.6 fmol/min and 414.6 ± 10.0 μM. The average computed catalytic efficiencies calculated on the basis of the proportion of IgG with catalytic activity (60.6 ± 60.3 M^{-1} min^{-1}) in the pool of polyclonal IgG showed a 477-fold difference with that of trypsin (28 922.3 M^{-1} min^{-1}), thus providing evidence for the lack of contamination by conventional serine proteases among the purified polyclonal IgG preparations. Based on the derived K_{cat} values, catalytic IgG cleaved 5–114 molecules of PFR-MCA in 24 h, indicating capacity of turnover.

Table 2
Quantity of catalytic IgG within the polyclonal pool of total IgG from patients with acquired hemophilia.

Patients' IgG	Quantity of PFR-MCA hydrolyzing IgG	
	Low hydrolyzing activity, pmol (%)	High hydrolyzing activity, pmol (%)
1	3.0 (56)	0.4 (8)
2	2.3 (43)	0.5 (10)
15	2.4 (44)	0.5 (10)
20	2.7 (51)	0.3 (7)
22	3.1 (59)	0.3 (6)
38	3.5 (66)	0.2 (3)
42	2.1 (40)	0.2 (5)
43	2.5 (48)	0.7 (14)
45	3.9 (73)	0.8 (15)
Mean ± S.D.	2.84 ± 0.58 (53 ± 11)	0.46 ± 0.21 (9 ± 4)

The values are expressed as pmoles of catalytic IgG per 5.3 pmoles of purified polyclonal total IgG. Values in round brackets represent the mean % of catalytic IgG with low and high activity. The results are representative of three individual experiments.

3.4. Evolution with time of the catalytic activity of IgG

We have demonstrated previously that 3/4 patients followed over the course of the disease had FVIII hydrolysis rates that co-evolved with inhibitory titers in plasma, suggesting that IgG-mediated FVIII hydrolysis participates in part in FVIII inactivation. Here, we followed eight patients over the course of the disease. Plasma was collected at the time of diagnosis and during the course of the disease for periods of time ranging from 6 to 140 days. At the time of diagnosis, inhibitory titers in plasma were 42, 6, 14, 2, 60, 56, 7 and 62 BU/ml, respectively (Table 1). At the time of the last sampling, all patients had detectable inhibitory titers of 18, 0.7, 3.5, 0.6, 30, 67, 1.6 and 19 BU/ml, respectively (Fig. 3). Thus, in the case of 7 patients, inhibitory titers had decreased by 2.0–8.6-folds, while one patient presented with a steady inhibitory titer (patient 28). IgG was purified from each of the plasma samples and assessed for serine protease activity towards PFR-MCA. At the time of diagnosis, hydrolytic activities of IgG from patients 9, 10, 11, 14, 25, 28, 32 and 35, were 1.08, 0.71, 0.55, 3.56, 5.14, 1.54, 7.17 and 3.31 mmol/min/mol, respectively (Table 1). At the time of the last sampling, the hydrolytic activities of patients' IgG were 1.72, 0.43, 0.79, 4.62, 0.84, 1.51, 2.99 and 1.51 mmol/min/mol (Fig. 3), respectively. Thus, in the case of 4 patients, the hydrolytic activity of IgG had decreased by 3.1 ± 2.0-folds. IgG from the remaining patients demonstrated stable (patient 28) or increased (patients 9, 11 and 14) hydrolytic activities. Overall, hydrolysis rates of PFR-MCA and Bethesda titers in plasma evolved following similar trends in the case of 6 patients (Fig. 3).

4. Discussion

While inhibition of FVIII by IgG-mediated hydrolysis is a feature shared by allo- and auto-antibodies to FVIII, FVIII-hydrolyzing alloantibodies presented heterogeneous FVIII hydrolysis rates,

Table 3
Kinetic parameters of the hydrolysis of PFR-MCA by IgG of patients with acquired hemophilia.

Patients/Control	V_{max}[a] (fmol/min)	K_m[b] (μM)	K_{cat}[c] (min^{-1})	Catalytic efficiency[d] $(M\ min)^{-1}$
1	46.1 ± 7.7	376.4 ± 169.8	0.013	38.5
2	74.9 ± 14.2	500.4 ± 90.9	0.027	53.6
15	111.9 ± 5.1	466.7 ± 33.2	0.039	83.1
20	112.5 ± 12.6	545.5 ± 152.5	0.037	69.4
22	20.1 ± 2.5	163.0 ± 38.1	0.004	24.0
38	31.8 ± 6.8	273.4 ± 178.7	0.005	21.1
42	189.5 ± 11.6	399.7 ± 118.4	0.079	209.6
43	41.0 ± 17.3	484.3 ± 391.5	0.013	31.2
45	44.0 ± 6.0	637.2 ± 170.0	0.009	15.2
Trypsin	201.4 ± 3.6	414.6 ± 10.0	12.0	28 922.3

[a,b] PFR-MCA was incubated at increasing concentrations (0–2 mM) with IgG (67 nM) of nine patients with acquired hemophilia or of trypsin (0.42 nM), for 24 h at 37 °C. Hydrolysis rates were computed as explained in Materials and Methods. The data were fitted to the Michaelis–Menten equation ($r^2 \geqslant 0.94$) and V_{max} and K_m were derived. The data represent the mean of three independent experiments.

[c] In the calculation of K_{cat}, in the case of patients' IgG, the quantity of catalytic IgG sites within the pool of IgG was estimated to be equal to 3.3 ± 0.8 pmol per 5.3 pmol (Table 2).

[d] The catalytic efficiency is the ratio of K_{cat} to the corresponding K_m.

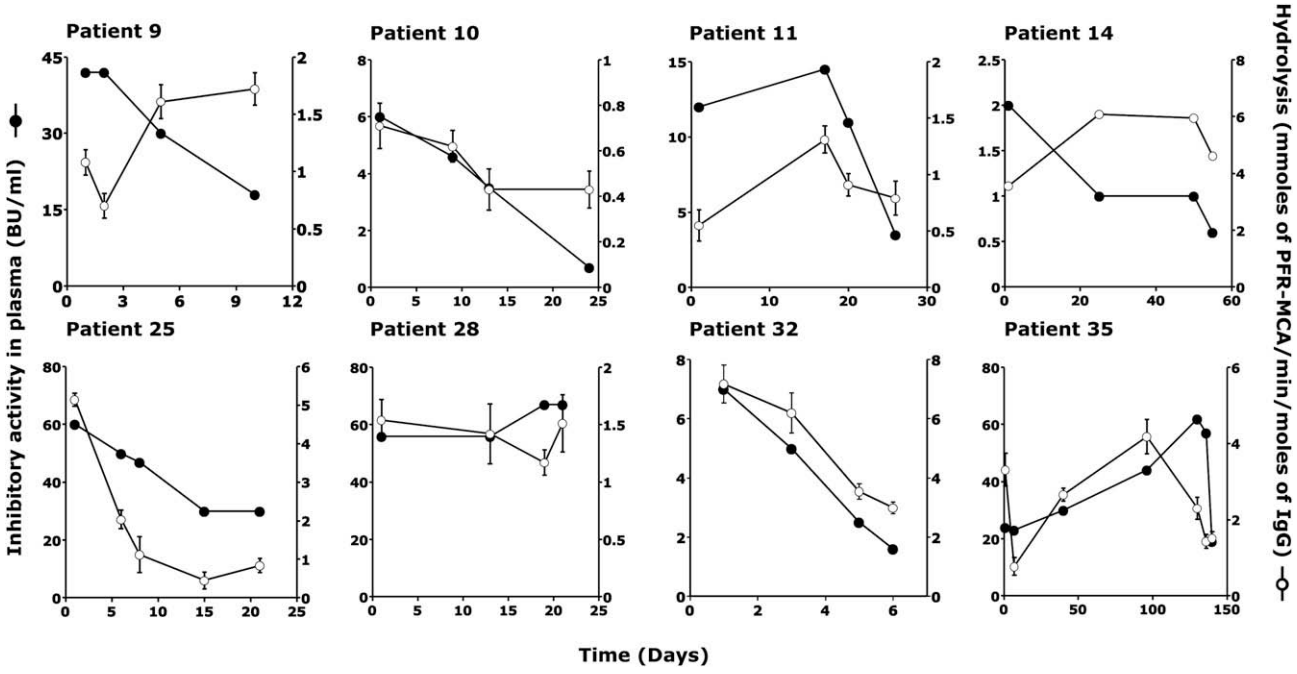

Fig. 3. Follow-up of the inhibitory and hydrolytic activities of patients' IgG. Plasma samples were collected from eight patients with acquired hemophilia during periods of time comprised between 6 and 150 days. Four to 7 samples were collected for each patient. For each sample, the inhibitory titer towards FVIII was measured in plasma (BU/ml, closed circle). IgG was purified from each plasma sample and assessed for PFR-MCA hydrolysis (mmol/min/mol, open circle). The graphs depict for each of the 8 patients the evolution with time of the inhibitory titer (left *y*-axis) and of the rates of IgG-mediated PFR-MCA hydrolysis (right *y*-axis).

with average rates that were greater and differed significantly from those measured for FVIII-hydrolyzing autoantibodies (Fig. 1E, $P = 0.033$). Previous studies have failed to distinguish FVIII inhibitory IgG that arise under autoimmune conditions from those that develop in an alloimmune context, with respect to the isotypic distribution and to the epitopes targeted on FVIII [3–5,10]. Anti-FVIII allo- and autoantibodies have however been found to differ in their kinetics of FVIII inactivation, and capacity to be neutralized by anti-idiotypic antibodies (reviewed in [11]), suggesting the existence of subtle differences in the epitopes recognized by the two groups of antibodies and in the structures of their variable regions. Whether such differences account for the differences in FVIII hydrolysis rates of allo- and auto-immune IgG remains hypothetical. Alternatively, the differences in proteolytic behavior of allo- and auto-antibodies may reflect the different immunological contexts under which FVIII-hydrolyzing IgG have been generated. In particular, while development of anti-FVIII alloantibodies may result from the inability of the patients' immune system to establish tolerance to a harmless exogenous antigen [12], appearance of anti-FVIII autoantibodies results from a break in tolerance to an endogenous molecule.

Our initial attempts to determine the kinetic parameters of FVIII-hydrolyzing IgG were hindered by the limited maximal concentration of FVIII available. To overcome this limitation, we have used a generic tri-peptide synthetic substrate, PFR-MCA [9], previously used to screen catalytic IgG, directed against antigen substrates such as vasoactive intestinal peptide [13] and thyroglobulin [14]. The cleavage of the amide bond between the amino acid arginine and the fluorescent moiety MCA justifies for a peptide bond hydrolysis, but may not essentially mimic the hydrolysis of a complex protein antigen. Indeed, alloimmune FVIII-hydrolyzing antibodies did not present a significant correlation between FVIII and PFR-MCA hydrolysis rates. Conversely, in the case of autoimmune FVIII-hydrolyzing IgG, and despite the

complex and varied etiologies that underly the development of autoimmune FVIII inhibitors, the FVIII hydrolysis rates correlated with PFR-MCA hydrolysis rates. The apparent V_{max} (74.7 ± 54.4 fmol/min) and average K_m (427.4 ± 143.6 μM) calculated for PFR-MCA hydrolysis in the case of IgG from nine patients with acquired hemophilia, differed from that previously published for five hemophilia A patients (12.7 ± 4.0 fmol/min and 846.6 ± 351.6 μM, respectively) [9], possibly accounting for the above mentioned discrepancy. However, the estimated K_{cat} (0.05 ± 0.02 min^{-1} in the case of alloantibodies [9] and 0.03 ± 0.02 min^{-1} in that of autoantibodies) and the average catalytic efficiencies (71 ± 32 and 61 ± 60 M^{-1} min^{-1}, respectively) did not allow to discriminate between the two groups of patients.

Recently, we described the co-evolution of the titers of IgG-mediated FVIII hydrolysis with the inhibitory titers measured in the plasma of acquired hemophilia patients during the course of the treatment. Here, we have performed a follow-up study in the case of eight patients. In the case of 6 out of 8 patients, we observed similar trends of co-evolution between the catalytic activity of IgG measured towards PFR-MCA and the measured Bethesda titers in plasma. The data demonstrate the reliability of PFR-MCA as a surrogate substrate for autoimmune FVIII-hydrolyzing IgG, and highlight the different natures of FVIII-hydrolyzing allo- and auto-antibodies.

Paul et al., reported that IgG from patients with systemic lupus erythematosis (SLE) and autoimmune thyroiditis (ATh), presented with significantly increased thyroglobulin-cleaving activities, as compared to IgG from healthy individuals or from patients with asthma or HIV infection [15]. Conversely, PFR-MCA hydrolysis was detected among IgG from healthy donors, but was extremely low or undetectable among IgG from SLE, ATh and rheumatoid arthritis patients [15,16]. Here, we document that IgG from alloimmune and autoimmune patients are endowed with greater levels of hydrolysis towards both FVIII and PFR-MCA, than IgG from healthy donors. Our results confirm that pathological conditions, whether alloimmune or autoimmune, are associated with increased levels of proteolytic IgG targeted towards cognate antigens. They however do not support the hypothesis of a universal occurrence of polyspecific catalytic activity in healthy donors that diminishes under autoimmune/inflammatory conditions.

We have previously reported on the absence of contaminating proteases among IgG purified from the serum of the different patients, that would account for the observed proteolysis of the different substrates [7]. Here, we have compared the data on the modification rates of purified catalytic IgG from patients to that of a conventional serine protease, trypsin. There was a 2.7-fold difference in the apparent V_{max} calculated between patients' IgG and trypsin (Table 3), while average K_m were in the same range. The catalytic efficiencies, calculated on the basis of the proportion of IgG with high catalytic activity in the polyclonal IgG pool, showed a 477-fold difference between patients' IgG and trypsin. Taken together, the data indicate that the modification rates calculated for purified IgG in our assay widely differ from that calculated for a conventional serine protease, thus suggesting that the architecture of the active site of FVIII-hydrolyzing molecules in our preparations is different from that of a conventional enzyme.

The occurrence of FVIII-hydrolyzing IgG has been reported in patients with hemophilia A [6], acquired hemophilia [7], sepsis [17] and renal transplantation [18]. FVIII-hydrolyzing IgG may thus be endowed with pathological roles; in inhibitor-positive patients with congenital hemophilia A, they inactivate therapeutic FVIII and hamper the control of hemorrhage. Alternatively, IgG-mediated FVIII hydrolysis may be beneficial in other pathological situations. Thus, patients with sepsis develop disseminated intravascular coagulation that may lead to organ failure [19]. Similarly, in patients with kidney transplant, uncontrolled activation of coagulation by the stressed endothelium of the graft is thought to play a role in the pathophysiology of chronic allograft nephropathy [20–22]. In both situations, IgG-mediated FVIII inactivation, within limits that are compatible with normal coagulation, may be beneficial by disrupting the amplification loop of thrombin generation which is dependent, at least in part, on factor VIII. The identification of the molecular immunological mechanisms underlying the selection of B lymphocytes secreting FVIII-hydrolyzing IgG under such a variety of pathological conditions remains the subject of intense research.

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Authorship and disclosures

Authors declare to have no conflict of interest.

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Reviews

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Review

Antibody-mediated catalysis: Induction and therapeutic relevance

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ABSTRACT

Abzymes are immunoglobulins endowed with enzymatic activities. The catalytic activity of an abzyme resides in the variable domain of the antibody, which is constituted by the close spatial arrangement of amino acid residues involved in catalysis. The origin of abzymes is conferred by the innate diversity of the immunoglobulin gene repertoire. Under deregulated immune conditions, as in autoimmune diseases, the generation of abzymes to self-antigens could be deleterious. Technical advancement in the ability to generate monoclonal antibodies has been exploited in the generation of abzymes with defined specificities and activities. Therapeutic applications of abzymes are being investigated with the generation of monoclonal abzymes against several pathogenesis-associated antigens. Here, we review the different contexts in which abzymes are generated, and we discuss the relevance of monoclonal abzymes for the treatment of human diseases.

Contents

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1. Introduction: A history of catalytic antibodies

One central concept in catalysis is the transition-state theory. Any chemical reaction may be viewed graphically in terms of free energy changes of the reactants as a function of a reaction coordinate. The most unstable chemical species appear at the highest point on the reaction coordinate, which is called the transition state. The rate of a chemical reaction is proportional to the free energy of activation which corresponds to the difference in free energy between that of the starting reactants and the transition state. Linus Pauling postulated that enzymes achieve catalysis because of their complementarity to the transition state for the reaction being catalyzed [1].

Antibodies are proteins produced by the immune system that bind and neutralize antigens. Each antibody binds to its molecular target similar to a key fitting in a lock or a substrate binding to an enzyme. In most cases, antibodies tightly bind the antigen, but do not specifically alter its chemical nature. The hypothesis proposed by Pauling in 1946 that the active center of an enzyme is targeted against the structure of the transition state, rather than to the native conformation of the substrate molecule [2], led Jencks in 1969 to propose that antibodies generated in an anti-hapten immune response against chemically stable analogs of the transition state of a reaction of interest could potentially display an enzymatic activity [3,4]. Indeed, in 1985, a general method for generating catalytic monoclonal antibodies (mAbs) against transition-state analogs, and a way to use those antibodies to accelerate chemical reactions, was first described. In 1986, using this approach, Richard Lerner and Peter Schultz independently reported antibody catalysis for the first time: monoclonal antibody-mediated hydrolysis of aryl esters and of carbonates, respectively [5,6].

Since 1986, catalytic antibodies, or abzymes, catalyzing more than 100 distinct chemical reactions have been obtained using TSA. Many catalytic antibodies achieve rate accelerations in the range 10^3 to 10^6. The rates of reactions catalyzed with abzymes, as measured by kinetic parameters such as K_M and V_{max}, are up to a million-fold greater than the corresponding uncatalyzed reactions. In many cases, however, catalytic antibodies have not yet approached the rates of reactions catalyzed by natural enzymes, owing either to the strong binding to the products of the reaction that greatly inhibits effectiveness, or to the difficulty to synthesize the proper immunogens.

This article reviews the different contexts in which abzymes may be generated, and discusses the relevance of monoclonal abzymes for the treatment of human diseases.

2. Strategies for deliberate induction of abzymes

2.1. Immunization with transition state analogs (TSA)

Making antibodies with binding pockets complementary to transition states is complicated by the fact that true transition states and most reaction intermediates are unstable. Thus, true transition states or intermediates cannot be isolated or used as haptens for immunization. Instead, so-called transition-state analog (TSA) molecules are used. Transition-state analog molecules are stable molecules that simply resemble a transition state (or intermediate) for a reaction of interest in terms of stereochemistry and charge distribution. To the extent that the transition-state analog molecule resembles a true reaction transition state or intermediate, the elicited antibodies will also be complementary to that transition state or intermediate and thus lead to the catalytic acceleration of that reaction (Fig. 1A). Abzymes catalyzing the hydrolysis of amides and esters, as well as reactions of cyclization [7–9], decarboxylation [10–12], lactonization [13], peroxidation [14], photochemical thymine dimer cleavage, bimolecular amide-bond formation, and other reactions not known to be catalyzed by natural enzymes have been described. Some abzymes have been produced that require cofactors for activity, similar to standard enzymes [15]. Later, in 1995 a new concept of "reactive immunization" emerged, wherein organisms are immunized with highly reactive haptens designed to make covalent link with the nucleophilic B-cell receptor (BcR) on the B cells, thus forming a TSA [16]. The catalytic efficiencies of aldolase abzymes, generated by reactive immunization, are only 40-fold lower than most studied aldolases [17,18].

2.2. Manipulation of the idiotypic network

Generation of abzymes using the idiotypic network was inspired by the "idiotypic network theory" proposed by Niels Jerne in 1974 [19]. According to this theory, immunization of an animal with an antigen results in the production of a 1st generation antibody (Ab1), with an antigen-binding site specific for an epitope on the antigen. Immunization with the variable region of the Ab1 induces the production of 2nd generation antibodies (Ab2), the antigen-binding domain of which is complimentary to the variable domain of Ab1 and, in some cases, possesses the internal image of the antigen epitope. If the initial antigen is an enzyme, some of the Ab2s will present with an internal image of the catalytic site of the enzyme, and will thus be catalytic (Fig. 1B). Manipulation of the idiotypic network has led to the generation of monoclonal abzymes with esterase activity [20], amidase activity [21], carboxypeptidase activity [22], protease activity [23] and recently allinase activity [24].

2.3. Immunization with electrophilic covalent reactive analog (CRA)

First reported in the year 2000, phosphonate diester molecules have been extensively used as a bait to fish-out antibodies with catalytic activity [25]. Covalent reactive analogs are haptenic phosphonates that bind covalently to a nucleophilic residue in the active site of an abzyme or of the BcR of the abzyme-producing B cells (Fig. 1C). S. Paul and co-workers have obtained monoclonal abzymes against the gp120 coat protein of HIV-1 virus using CRA-coupled gp120 as an immunogen [26], and against the amyloid-β peptide by screening an scFv library against a CRA-derivatized amyloid-β peptide [27]. Likewise, Tramontano and colleagues have successfully used covalently reactive phosphonate esters to isolate catalytic scFv from a semi-synthetic scFv phage display library [28].

2.4. Immunization with ground state antigens

Immunization of normal or autoimmune-prone mice with ground state antigens has allowed to generate several monoclonal abzymes with catalytic activities against vasoactive intestinal peptide [29], CCR5 [30], HIV-1 coat protein gp41 [31], gp120 [32], *Helicobacter pylori* urease [33] and TNFα [34].

2.5. De novo generation of abzymes by site-directed mutagenesis of antibodies

De novo elicitation of catalytic activity in antibodies has been performed by introduction of amino acid residues with known catalytic functions into the antigen-binding site of antibodies (Fig. 1D). This approach is advantageous because the antibody, which is to be modified into an abzyme, is of a predetermined specificity. Fletcher et al. were able to introduce ribonuclease activity in a scFv variant of a Fab, which was specific for RNA [35]. In another instance, Liu et al. were able to induce protease activity in a scFv specific for the bacterial protein HPr. Introduction of 4 mutations in the antigen-binding domain resulted in induction of protease activity with no loss in specificity for HPr [36]. In a similar approach, peptidase activity has been introduced into a light chain expressed at the yeast cell surface, by substituting two residues Ser27a and Asp1. The substitutions

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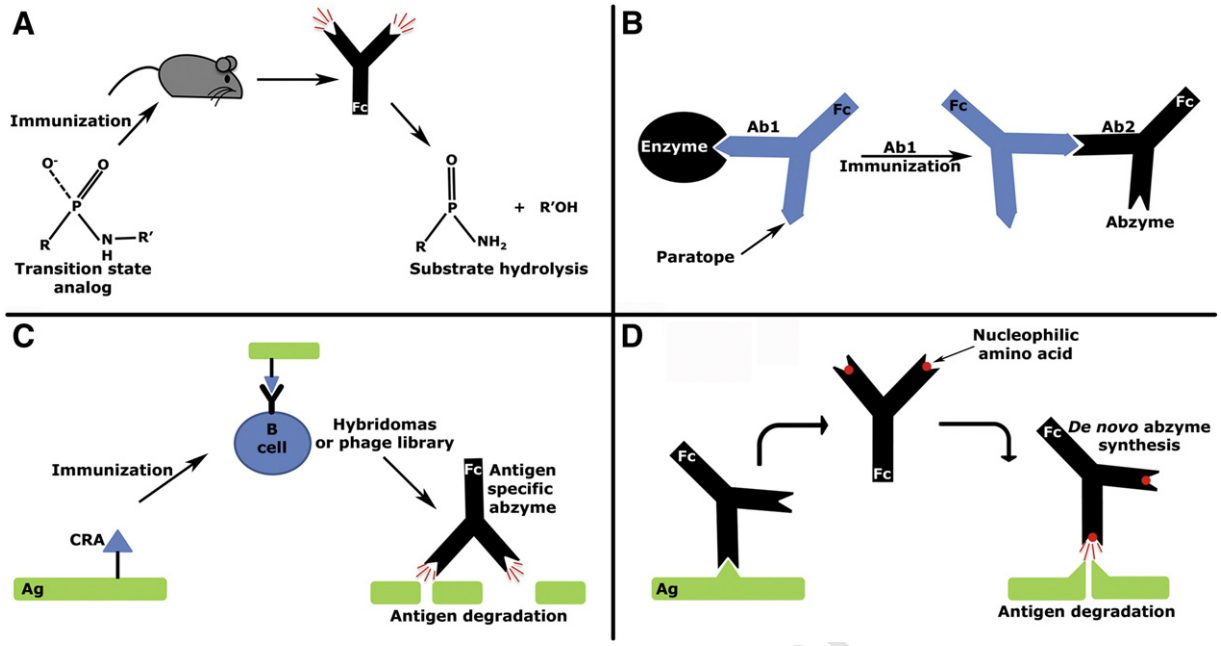

Fig. 1. Different methods to generate monoclonal abzymes. A. Stable transition state analogs (TSA) of known chemical reactions are synthesized and used as haptens to immunize mice. Monoclonal antibodies are selected based on high binding to the TSA. Some of the high binders exhibit good complementarity with the transition state, thus enabling the acceleration of catalysis when incubated with the substrate. B. Abzyme generation by manipulation of the idiotypic network requires generation of a neutralizing antibody (Ab1) that is directed against the active site of the enzyme (E) of interest. In the course of immunization, the paratope of the Ab1 provides an inverse image of the active site of the enzyme, which acts as a template for synthesis of the mirror image of the active site in the anti-idiotypic antibody (Ab2). Ab2s with an enzymatic activity similar to that of the enzyme are referred to as Abzymes. C. An antigen (Ag) coupled to a covalent reactive analog (CRA) of a reaction-based enzyme inhibitor is injected in mice. The spleen is isolated to generate hybridomas from the B cells or to construct a library of scFv-expressing phages. The catalytic B cells or scFvs are then selected based on covalent binding to the CRA-coupled antigen. D. Catalytic activity can be conferred *de novo* to a non-catalytic monoclonal antibody, by introduction of a serine protease-like catalytic triad in the paratope of the antibody.

were done for the elicitation of a serine protease-like catalytic triad with a His93; already present within the CDR [37].

2.6. Isolation of abzymes by targeting specific germline genes

Several abzymes possess variable region-encoding genes in the germline configuration [38]. In line with this observation, the presence of the Asp1, Ser27a and His93 catalytic triad was investigated in mouse kappa light chain of a total of 84 clones by the group of Uda: pseudo catalytic triads were found mainly in the bb1, cr1, cs1, bl1, bj2 and bd2 germline kappa genes [39]. In parallel, the same group screened genes encoding human kappa light chains, upon structural prediction using a strict spatial arrangement of the catalytic residues Ser, His and Asp. Nine germline genes belonging to subgroup II showed a high prevalence of pseudo catalytic triads. Cloning and expression of these genes from circulating B cells of a healthy individual vaccinated against rabies virus, allowed the generation of virus-specific abzymes [40].

3. Abzymes as therapeutic tools

The antibody scaffold provides a unique combination of antigen specificity and functional activity to generate novel enzymes. Nowadays, attempts are made to take advantage of these synergistic properties for the treatment of human diseases.

3.1. Abzymes against infectious agents

Several virus-specific abzymes have been developed in the recent years. Thus, the HIV-1 coat proteins gp120 and gp41 have been targeted because of the key role they play in infecting CD4+ T lymphocytes. Three monoclonal abzymes able to cleave gp120 in a site-specific manner were generated upon immunization of mice with a CRA-coupled gp120 [26]. Because HIV-1 is known to escape from the host immune system by undergoing repeated mutations in its coat proteins, abzymes were also generated from a phage library established from lupus patients that cleaved the conserved superantigenic region 421–433 [41]. In parallel, the group of Uda has generated abzyme light chains by ground state immunization of the conserved region of gp41 [31].

Catalytic kappa light chains have been isolated from an individual vaccinated against rabies virus; the light chains cleaved small peptide substrates and, in some cases, diminished the infectivity of rabies virus (CVS-11) in mouse NA cells and protected mice upon intracerebral inoculation with CVS-11 [40].

Lastly, the group of Uda has isolated a light chain abzyme (UA15-L) specific for the urease of *H. pylori* [33]. UA15-L was able to hydrolyze both recombinant UreB and the whole urease, and degraded urease in the intact bacterium, thus significantly decreasing colonization by *H.* pylori in the stomach of mice.

3.2. Abzyme prodrug therapy

The first documentation of abzyme-mediated activation of a prodrug was provided in 1993, when Miyashita et al. showed the activation of a non-bioactive chloramphenicol ester by an abzyme generated against a phosphonate TSA [42]. Later in 1996, Wentworth et al. generated an abzyme that activated the carbamate prodrug into its cytotoxic form, nitrogen mustard, that was able to decrease the viability of human colonic carcinoma cells *in vitro* [43]. The most studied abzyme for prodrug therapy is 38C2 that was generated by reactive immunization with the 1,3 diketone hapten, and possesses an aldolase activity [17]. 38C2 activates the prodrug form of the anti-cancer drugs doxorubicin and camptothecin [44]. It was shown to inhibit the growth of human colon carcinoma and prostrate cancer cell lines and to reduce tumor growth in an animal model of neuroblastoma [45]. 38C2 also showed therapeutic potential in diabetes upon activation of an aldol-modified insulin [46]. More recently, a catalytic nanobody with alliinase activity was obtained upon manipulation of the idiotypic network. The abzyme nanobody suppressed

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the growth of the B16 tumor cell line by converting the precursor prodrug alliin into cytotoxic allicin [24].

3.3. Abzymes in inflammation

A potential beneficial role of abzymes has been investigated in Alzheimer's disease by virtue of their capacity to proteolytically degrade amyloid aggregates. Proteolytic cleavage of amyloid-β by recombinant light chains [47], heterodimeric light chains and single chain abzymes [27] was documented with exceptionally elevated hydrolysis rates. It was proposed that single chain variant abzymes may combine efficient Aβ clearance and prevention of Fc-mediated inflammation caused by phagocytic cells.

A monomeric heavy chain abzyme specific for the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) was recently developed upon immunization with the ground state molecule [39]. In the context of the treatment of autoimmune and inflammatory diseases by anti-TNF therapy [48–51], the capacity of abzymes to turnover should allow to diminish the requirement for bolus doses of therapeutic antibodies that are usually required for the clearance of TNF-α.

3.4. Abzymes for gene silencing

Gene silencing by targeting mRNA has been achieved by conventional small interfering RNA (siRNA) technique. Alternatively, inhibition of intracellular proteins by cell penetrating antibodies (TransMabs) is also being investigated [52]. In a novel approach, Lee et al. have achieved gene silencing by abzymes specific for the mRNA of Her2, a protein that is overexpressed in breast cancer cells [53]. The abzymes are variants of a DNA/RNA-hydrolyzing antibody light chain 3D8, obtained by generating a synthetic library by randomization of amino acids. The abzyme variants selectively hydrolyzed the Her2 mRNA, resulting in decreased surface expression of Her2 and induction of apoptosis, with a cytotoxic potential greater than that of siRNA.

3.5. Abzymes against coagulation factors

We have demonstrated in the past the hydrolysis of coagulation factor VIII (FVIII) by IgG purified from the serum of patients with congenital and acquired hemophilia A [54–58], suggesting that catalytic antibodies may interfere with hemostasis under pathological conditions. Interestingly, IgG-mediated catalytic activity was higher in the plasma of patients who survived septic shock as compared to those who deceased [59]: indeed, IgG from some patients hydrolyzed FVIII and factor (FIX), potentially modulating disseminated vascular thrombosis. Similar observations were seen in patients with chronic allograft nephropathy (CAN) where IgG from patients' plasma were shown to hydrolyze FVIII and FIX. Patients with higher IgG-mediated catalytic activity had better graft survival, and low catalytic activity measured at 3 months post-transplantation was predictive of CAN 2 years later [60].

In contrast to these observations where the IgG-mediated hydrolysis of coagulation factors is presumed to hamper coagulation, we have recently described that hydrolytic IgG from some patients with acquired hemophilia activates## FIX and restore thrombin generation *in vitro*, provided that a basal level of FVIII is present in the assay [61]. The presence of FIX-activating catalytic IgG tended to be significantly associated with patient survival 12 months following diagnosis. Although the measured kinetics of IgG-mediated FIX activation are low, this may be compensated by the long half-life and substantial concentration of IgG in the blood. Thus, a fraction of the circulating FIX upon IgG-mediated activation may "feed in" the coagulation cascade and compensate for the loss of FVIII activity in patients with acquired hemophilia.

4. Conclusion

Abzymes appear as a double-edge sword. They have been described in a number of human diseases, including autoimmune disorders, alloimmune responses to protein therapeutics, and viral or bacterial infections [61]. However, it is unclear as yet whether catalytic antibodies that develop in pathological conditions play a pathogenic role or reflect an attempt of the organism to re-establish homeostasis. Conversely, monoclonal abzymes demonstrate an extraordinary potential as a novel class of therapeutic molecules. They combine high substrate/antigen specificity, with turnover, relatively low catalytic efficiency and elevated half-life. In certain cases, expression of the variable catalytic domains in the absence of the Fc portion may advantageously reduce risks for activation of the inflammatory machinery. It is anticipated that the recently developed strategies to generate highly proficient abzymes will have implications in close future for the treatment of infectious, alloimmune and autoimmune disorders.

Take-home messages

- Abzymes are antibodies that behave like enzymes.
- Abzymes are found under pathological conditions, although their pathogenic or beneficial role remains unclear.
- Abzymes with pre-defined specificity and activity may be generated *a façon* using an increasing number of biotechnological approaches.
- By virtue of combining high substrate/antigen specificity, enzyme-like turnover, relatively low catalytic efficiency and elevated half-life, abzymes reveal as promising therapeutic tools for human disorders.

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Do proteolytic antibodies complete the panoply of the autoimmune response in acquired haemophilia A?

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Summary

Acquired haemophilia A (AHA) is a rare bleeding disorder characterized by the sudden generation of autoantibodies against factor VIII (FVIII) in individuals with no previous history of abnormal haemostasis. Understanding the pathogenesis of this disease has been hampered by the rarity of the patients and the difficulty in obtaining biological material from untreated patients. Still, progress has been made recently in understanding the pathogenesis of AHA. In particular, the importance of $CD4^+$ T cells in AHA development has been documented and the epitopes targeted by T cells on FVIII have been delineated. Accordingly, a polymorphism in the cytotoxic T-lymphocyte-associated protein 4 gene (*CTLA4*), known to participate in the regulation of $CD4^+$ T-cell responses, and a preferential usage of certain human leukocyte antigen class II haplotypes, have been associated with the disease. Recent findings have documented the presence of immunoglobulin G (IgG) with proteolytic activity against FVIII and factor IX (FIX) in patients with AHA. While FVIII-hydrolysing IgG has been shown to inactivate FVIII, FIX-hydrolysing IgG from AHA patients activate FIX *in vitro*. Here, we describe the latest findings on the immunopathogenesis of AHA, with a special focus on the potential role played by antibodies endowed with proteolytic properties.

Keywords: Acquired haemophilia A, factor VIII, factor IX, factor VIII inhibitors, catalytic antibodies.

Acquired haemophilia A (AHA) is a bleeding disorder that occurs following the development of a neutralizing autoimmune response against the endogenous coagulation factor VIII (FVIII). AHA occurs at an overall incidence rate of 1·48 per million/year and it mainly affects the elderly, with an incidence rate as high as 14·7 per million/year seen in patients above 85 years (Collins *et al*, 2007). The clinical feature of AHA is the occurrence of sudden bleeding episodes in individuals without previous bleeding history. Bleeding is life threatening in about 80% of the cases, especially in the early stage of the disease or in cases of excessive bleeding following trauma, surgery or cerebral haemorrhages (Collins *et al*, 2007). The bleeding patterns in AHA include a high prevalence of haemorrhages in skin, muscle or soft tissue and mucous membrane (Green & Lechner, 1981; Boggio & Green, 2001).

Acquired haemophilia A is a disease with a complex aetiology. In 50% of cases, AHA is not associated with any pertinent disease, while the remaining cases of AHA are associated with autoimmune diseases (17–18% of patients) (Green & Lechner, 1981; Yee *et al*, 2000), post-partum (7–21%) (Franchini, 2006), solid cancers, monoclonal haemopathies, skin disorders, use of drugs, infection and even vaccination. While much information on the clinical aspects is available, a thorough dissection of AHA pathogenesis at the cellular and molecular levels has been challenging due to limited access to biological samples. However, an appreciable progress has been made in recent years and the properties of the inhibitory anti-FVIII autoantibodies have been delineated both at the functional and structural levels. Similarly, the implication and epitope specificity of $CD4^+$ T lymphocytes have been studied in detail. More recently, a few polymorphisms in genes implicated in the control of the immune response have been associated with the occurrence of AHA. Further, interesting findings in AHA on the pathogenic and beneficial role of antibodies with proteolytic properties have shed light on new parameters of auto-immune response. In the present review, we describe the latest findings on the immunopathogenesis of AHA, with a special focus on the potential role played by proteolytic antibodies.

Immune response to FVIII in AHA

Immune responses to FVIII

Immune responses to FVIII are found in three different immunological contexts. Anti-FVIII allo-antibodies develop in

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some patients with congenital haemophilia A following replacement therapy with exogenous FVIII (Ehrenforth *et al*, 1992). This allo-immune response is believed to reflect a classical immune response to a foreign antigen, although it remains unclear why some patients develop an immune response to FVIII while others do not, and why some patients develop an immune response at all, given the fact that therapeutic FVIII is administered intravenously, a notoriously non-immunogenic route of antigen administration.

Acquired haemophilia A results from the spontaneous development of autoantibodies against endogenous FVIII. The context of the initiation of the autoimmune response to self-FVIII is not understood. We believe that two different immune contexts may pre-exist for the development of autoimmune FVIII inhibitors, which will be addressed below.

Lastly, anti-FVIII antibodies of the immunoglobulin G (IgG) isotype have been described in healthy individuals (Algiman *et al*, 1992; Moreau *et al*, 2000); these antibodies are referred to as natural anti-FVIII autoantibodies and have been shown to inhibit the pro-coagulant activity of FVIII *in vitro* following purification from serum by affinity-chromatography (Moreau *et al*, 2000). *In vivo*, natural anti-FVIII autoantibodies are neutralized by the presence of anti-idiotypic antibodies that control their inhibitory potential towards FVIII (Rossi *et al*, 1988; Kazatchkine *et al*, 2000). Accordingly, natural FVIII-reactive $CD4^+$ T cells are found within peripheral blood mononuclear cells of healthy donors, which are controlled by the presence of regulatory $CD4^+CD25^+$ T cells (Kamate *et al*, 2006).

We shall summarize below the information that is currently available regarding the involvement of $CD4^+$ T cells in the pathological autoimmune anti-FVIII response and will describe the properties of FVIII inhibitors found in patients with AHA.

Requirement for T-cell help in the anti-FVIII autoimmune response

A classical immune response to an antigen requires several sequential steps: the antigen is endocytosed by antigen presenting cells (APCs), such as dendritic cells or macrophages. The APCs process the antigen and present antigen-derived peptides at their surface in complex with major histocompatibility complex (MHC) class II molecules. The peptide-MHC complexes are recognized by the T-cell receptor (TcR) of $CD4^+$ T lymphocytes. If T cells simultaneously receive co-stimulatory signals from the APCs, they are activated and differentiate into effector T cells that will then assist antigen-specific B lymphocytes. In turn, the antigen-specific B lymphocytes are activated, differentiated into memory cells or into plasma cells that produce the antibodies. A strong T-cell dependent immune response generally leads to the production of high affinity antigen-specific antibodies of the IgG isotype.

As mentioned above, natural anti-FVIII IgG and natural FVIII-reactive $CD4^+$ T cells are found in healthy individuals. This demonstrates that, under physiological conditions, endogenous FVIII is internalized by APCs and presented to T cells that receive strong enough co-stimulatory signals to assist B cells. However, regulatory T cells that control natural autoreactive anti-FVIII T cells have also been identified (Kamate *et al*, 2006). In patients with AHA, the autoimmune response results from a break of tolerance towards self-FVIII. The requirement for T cells in the pathogenesis of AHA probably depends on the underlying disorder, which precedes the emergence of AHA.

In the literature, a few cases of AHA have been associated with monoclonal gammapathies, as seen in patients with Waldenström macroglobulinemia (Castaldi & Penny, 1970) or myeloma (Glueck & Hong, 1965; Sallah *et al*, 2000; Holme *et al*, 2005; Sari *et al*, 2009). It is probable that FVIII inhibitors in these patients do not result from the development of a classical immune response, but from the stochastic 'tumourogenization' of B-cell clones that encode FVIII-specific immunoglobulins that may be of the IgM, IgG or IgA isotypes (Glueck *et al*, 1989). In the large majority of AHA patients however, the immune response to FVIII is polyclonal and is characterized by the production of polyclonal IgG. In these patients, the disease is idiopathic or associated with autoimmune, inflammatory or infectious conditions, or develops post-partum. The reasons for which an innocuous self-antigen is suddenly 'seen' by the immune system as foreign and 'undesired', are not clear. As for most autoimmune diseases, mimicry with foreign (viral or bacterial) antigens may evoke immune dysregulation or excessive inflammation. Concrete evidence is however scarce. The available literature suggests that, under these conditions, $CD4^+$ T cells play a role in the pathogenesis of the disease.

Reding *et al* (1999, 2000) dissected the specificity of FVIII-reactive $CD4^+$ T cells in patients with AHA. These authors isolated circulating mononuclear cells from the blood of about 10 patients with AHA. The cells were incubated with human recombinant FVIII or with arrays of peptides spanning the heavy and light chains of FVIII. The *in vitro* proliferation of the memory $CD4^+$ T cells was assessed upon incorporation of tritiated thymidine (Reding *et al*, 1999, 2000). Memory $CD4^+$ T cells from AHA patients were shown to proliferate in the presence of intact FVIII. The use of overlapping peptides demonstrated proliferation of the T cells against peptides originating from the A2, A3 and C2 domains (Reding *et al*, 2000). More detailed analyses indicated that peptides from the C2 domain, spanning the amino acids 2191–2210, 2241–2290 and 2291–2330, were the most prone to induce $CD4^+$ T cell proliferation. Interestingly, these peptide regions are also known to overlap with inhibitor-binding sites (Reding *et al*, 2003). T cells from healthy individuals also proliferated in response to the peptide spanning amino acids 2241–2290. In the case of the A3 domain, a peptide spanning the amino acids 1831–1850 was found to significantly induce the proliferation of T cells from AHA patients (Reding *et al*, 2004).

Anti-FVIII autoantibodies in AHA

Fulcher *et al* (1987) reported the presence of FVIII-specific antibodies of the IgG1 and IgG4 subclasses in 5 patients with AHA (Fulcher *et al*, 1987). Further, a more recent study documented the presence of inhibitory IgG4 antibodies in 18 patients with AHA (Scandella, 1999). Additionally, Reding *et al* (2002) documented a correlation between high levels of anti-FVIII IgG4 antibodies and elevated inhibitory titres. Conversely, at least half of the AHA patients with low inhibitor titres, predominantly had IgG1 and IgG2 anti-FVIII autoantibodies in this study (Reding *et al*, 2002).

The available information regarding the *IGHV* genes that encode inhibitory anti-FVIII autoantibodies in AHA comes from studies by the group of J Voorberg (van den Brink *et al*, 2000a,b, 2001, 2002). In this work, several scFv libraries were generated using the circulating memory B cells of patients with mild and severe haemophilia A, as well as from one patient with AHA. In their experimental set-up, the patients' IgG4-specific *IGHV* gene repertoire was amplified and combined with a variable light chain (*IGLV*) gene repertoire of non-immune origin. The scFv displayed at the surface of filamentous phages were then selected for binding to different domains of the FVIII molecule. In the case of the only AHA patient studied, four clones were isolated that bound to the C2 domain of FVIII. The isolated scFv clones had *IGHV* genes encoded by the germline gene segments DP-10, DP-14 and DP-88, that all belong to the *IGHV1* gene family. The clones harboured extensive hypermutations (11–16 amino acid substitutions), indicating that the autoimmune anti-FVIII humoral response in AHA undergoes affinity maturation, indirectly confirming the help provided by FVIII-specific $CD4^+$ T cells. Interestingly, other monoclonal C2-specific anti-FVIII IgG have been derived either from a scFv library prepared from cells of a patient with mild haemophilia A (van den Brink *et al*, 2002), or from the Epstein–Barr virus-transformed memory B cells of a patient with severe haemophilia A (Jacquemin *et al*, 1998); all anti-C2 antibodies were shown to be encoded by *IGHV* genes that were also homologous to the *IGHV1* gene family. Whether all C2-specific IgG in AHA are encoded by *IGHV1* genes remains to be confirmed upon analysis of a large repertoire of patients. Such a result would pave the way towards the development of therapeutic approaches aimed at specifically targeting B cells expressing restricted families of *IGHV* genes, for instance using anti-idiotypic strategies (Kazatchkine *et al*, 2000; Gilles *et al*, 2004).

Epitope specificity of anti-FVIII autoantibodies in AHA

Available data on the epitope specificity of FVIII inhibitors show that the main target domains for anti-FVIII IgG of patients with AHA are the A2 and C2 domains, while the A1, A3 and C1 domains are rarely targeted. In a cohort of 21 AHA patients, Prescott *et al* (1997) showed that antibodies from 13 patients were exclusively directed against either the C2 or the A2 domain with a majority (IgG from 10 patients) being directed to the C2 domain. Another study, which included 22 AHA patients (Scandella, 1999), showed a mixed specificity of autoantibodies towards the A2 and C2 domains. Importantly, high inhibitory titres have been associated with recognition of either the A2 or C2 domains of FVIII. This is in agreement with similar studies performed on inhibitor-positive patients with congenital haemophilia A.

As was reported in the case of anti-FVIII alloantibodies found in patients with congenital haemophilia A; C2 domain-specific autoantibodies in AHA patients inhibit the binding of FVIII either to von Willebrand factor (VWF) or to phospholipids, thus preventing the protective effect that VWF plays on FVIII catabolism or the role played by phospholipids in FVIII activity (Gawryl & Hoyer, 1982; Arai *et al*, 1989). Further, antibodies from AHA patients, similar to those from patients with congenital hemophilia A, may also inhibit the activation of FVIII by thrombin and activated factor X; as shown by their ability to prevent the binding of C2 domain-specific non-classical (type II inhibitors) antibodies (Meeks *et al*, 2007, 2008). Lastly, binding of anti-FVIII autoantibodies to the A2 and A3 domains prevents the interaction of FVIII with thrombin, activated factor IX and/or factor X, thus hampering the formation of the tenase complex (Fay & Scandella, 1999; Scandella, 1999; Gharagozlou *et al*, 2009).

Kinetics of FVIII inhibition

Anti-FVIII inhibitory antibodies have been classified into type I and II antibodies, based on their kinetics of FVIII inactivation. Antibodies with type I kinetics inhibit the pro-coagulant activity of FVIII in a dose-dependent manner; in other words, the amount of FVIII activity measured in the Bethesda assay is proportional to the dilution of the plasma tested. In contrast, type II antibodies exhibit non-linear inactivation patterns, and total FVIII inactivation is generally not reached even when plasma is tested undiluted. A preventive role for VWF has been attributed to the partial inactivation of FVIII by type II inhibitors. Thus, it was demonstrated that some type II inhibitors compete with VWF for binding to FVIII (Gawryl & Hoyer, 1982), although this is not the case for all type II inhibitors (Jacquemin *et al*, 2000). In rare cases, VWF has been shown to be required to allow certain type II antibodies to exert their inhibitory activity, by reducing the rate of dissociation of activated FVIII from VWF (Peerlinck *et al*, 1999). Interestingly, while alloantibodies from patients with congenital haemophilia A generally behave like type I antibodies, autoantibodies to FVIII more frequently exhibit type II kinetics of FVIII inactivation. Rather than differences in epitope specificities, differences in binding affinities for FVIII may be the discriminatory feature between type I and II inhibitors.

Risk factors for AHA

The development of autoimmunity represents a breakdown in the balance between immunogenic and tolerogenic signals that constantly trigger the immune system. The fine-tuning of this balance is under the control of multiple factors. In this respect, the onset of AHA probably results from a combination of genetic and environmental factors, together with a decline in immune tolerance that is associated with ageing, or that accompanies the postpartum period. A couple of genes have been proposed to be associated with the development of AHA; these include genes encoding HLA molecules and some co-stimulatory molecules that are essential in the cross-talk between antigen presenting cells and $CD4^+$ T lymphocytes.

Polymorphisms in CTLA4 in AHA patients

The cytotoxic T-lymphocyte-associated protein 4 *(CTLA4)* gene is an important co-stimulatory molecule present at the surface of activated $CD4^+$ T lymphocytes and of regulatory T cells. It is involved in the preservation of T-cell homeostasis and peripheral tolerance by down-regulating effector T-cell responses. Interestingly, two studies have demonstrated the association of single nucleotide polymorphisms (SNPs) in the *CTLA4* gene with inhibitor development in patients with congenital severe haemophilia A (Astermark *et al*, 2007; Pavlova *et al*, 2009).

The frequencies of different SNPs in *CTLA4* have been reported in AHA patients (Pavlova *et al*, 2008). Genetic polymorphisms of *CTLA4* were studied at three positions: −318 C/T, +49 A/G and CT60 A/G. The *CTLA4* 49 A/G polymorphism occurred at a significantly higher frequency in patients with AHA compared with controls. A sub-cohort analysis showed a higher frequency of the G allele in groups of patients with idiopathic AHA and in AHA patients with underlying autoimmune diseases (Pavlova *et al*, 2008). The *CTLA4* 49 A/G polymorphism has been shown to change a threonine into an alanine at position 17 of the leader peptide sequence (Ligers *et al*, 2001; Maurer *et al*, 2002). This alteration causes an improper compartmentalization of the molecule in the endoplasmic reticulum, resulting in hampered mobilization of the molecule at the cell surface. The ensuing decreased expression of CTLA4 has been shown to hamper the CTLA4-driven down-regulation of T-cell activation.

The effect of the *CTLA4* polymorphism has not been shown at the protein level in AHA patients. However, with reference to other studies (Ligers *et al*, 2001; Maurer *et al*, 2002), we may speculate that AHA patients bearing such polymorphisms have inadequate peripheral tolerance towards endogenous FVIII. This would result in an inappropriate control of FVIII-specific $CD4^+$ T cells, leading to facilitated B-cell activation and development of inhibitors.

Of interest, seminal work by Astermark *et al* (2006a,b) have documented strong associations between a SNP in the *TNF* and *IL10* genes, with inhibitor development in severe congenital haemophilia A patients. Both polymorphisms had been previously associated with increased transcription and secretion of the respective cytokine, thus leading to inflammation in the case of TNF, or to B-cell activation in that of IL10 (Wilson *et al*, 1997; Zheng *et al*, 2001). To our knowledge, associations between polymorphisms in *TNF* and *IL10* and the onset of AHA have not been reported as yet.

HLA haplotypes in AHA patients

A recent study including 57 AHA patients investigated associations between the development of the disease and HLA class I and class II haplotypes (Pavlova *et al*, 2010). While there was no association of AHA with HLA class I haplotypes, a significantly higher frequency of the HLA class II molecules DRB1*16 and DRB1*0502 was found in AHA patients as compared to healthy controls. In contrast, the HLA class II molecules DRB1*15 and DQB1*0602 were detected at significantly lower levels. Interestingly, a similar analysis performed in a population of inhibitor-positive and inhibitor-negative patients with severe congenital haemophilia A yielded completely opposite results (Pavlova *et al*, 2010). The alleles found to be susceptible in AHA were identified as low inhibitor risk alleles in congenital haemophilia A. Conversely, DRB1*15 and DQB1*0602, identified as protective alleles in AHA, were found to be high inhibitor risk alleles in congenital haemophilia A (Pavlova *et al*, 2009). DRB1*15 is particularly suited to the presentation of the FVIII-derived peptide 1706–1721 to $CD4^+$ T lymphocytes, and has been proposed to participate in the alloimmunization process against exogenous FVIII in severe haemophilia A (Pavlova *et al*, 2009). It is tempting to speculate that, in AHA, high affinity HLA alleles such as DRB1*15 are mostly involved in conferring tolerance to the endogenous FVIII; the DRB1*15 HLA class II allele would play a major role in presenting processed FVIII peptides to newly arising T cells in the thymus, thus favouring the elimination of autoreactive FVIII-specific $CD4^+$ T lymphocytes and the generation of regulatory T cells in the thymus or at the periphery.

Polymorphisms in the F8 gene in AHA patients

One intriguing field of study regarding genetic predisposition to AHA is related to particular polymorphisms in the FVIII gene (*F8*). Indeed, a novel SNP C.8899G > A was found at the 3′UTR region of exon 26 in two Korean patients with AHA (Hwang *et al*, 2011). While this has not been reported by other groups in the case of AHA as yet, a similar correlation between a SNP in the 3′UTR of *F9* and levels of circulating factor IX in haemophilia B has been proposed (de la Salle *et al*, 1993). More recently, several polymorphisms have been reported in the coding sequence of *F8*, which are differently distributed between patients of different ethnic origins. Whether particular combinations of the different SNPs in *F8* facilitates the development of pathogenic autoimmune responses to FVIII remains to be investigated (Viel *et al*, 2009).

Proteolytic activity of antibodies in AHA

Generation of catalytic antibodies

Catalytic antibodies are immunoglobulins endowed with enzymatic activities. This possibility was first postulated by Pauling (1946), and demonstrated 30 years later (Satterthwait & Jencks, 1974). The first molecular analysis of catalytic antibodies became possible in the 1980's with the production of the monoclonal antibodies using hybridoma technology (Pollack *et al*, 1986; Tramontano *et al*, 1986). The catalytic activity of immunoglobulins is associated with the antigen-binding site, which has been shown to mimic structurally and functionally the active site of enzymes (Fig 1).

Several experimental protocols have been used to generate monoclonal catalytic antibodies (for review, see Lacroix-Desmazes *et al*, 2006a). Historically, the first attempts were performed upon immunization of mice with transition-state structural analogues of substrate molecules, which represent chemical structures, as seen in enzyme-catalysed reactions (Thomas, 1994). Alternative strategies have been based on the use of covalent reactive analogues (CRA) of suicide inhibitors for serine proteases, such as phosphonate diesters. The CRA molecules bind covalently to the active site serine residue after engaging into a nucleophilic-electrophilic electron attack. As a bait, the CRA moieties are coupled to the amino acid corresponding to the cleavage site on the target antigen/substrate, and the CRA-peptide chimera are used to immunize mice or screen phage libraries for fishing out catalytic antibodies (Paul *et al*, 2003; Taguchi *et al*, 2008). In an original approach, one group has exploited the idiotypic network to generate monoclonal catalytic antibodies. The 'idiotypic approach' first requires the generation of a monoclonal antibody, referred to as Ab1, which binds to the active site cleft of the enzyme of interest. Secondary monoclonal antibodies, referred to as Ab2, are then generated against the variable region of the Ab1. While the antigen binding site of the Ab1 represents an 'inverse' image of the active site of the enzyme, some of the generated Ab2 carry an internal image of the active site of the enzyme, and are thus endowed with similar enzymatic activities: antibodies with esterase, lactamase and amidase activities have thus been produced (Izadyar *et al*, 1993; Avalle *et al*, 1998; Ponomarenko *et al*, 2007).

Catalytic antibodies in physiological and pathological conditions

Catalytic antibodies also arise spontaneously in humans, without deliberate immunization. The first description was provided by Paul *et al* (1989) who documented the presence of IgG with the ability to hydrolyse the vasoactive intestinal peptide in patients with asthma. A few years later, the presence of DNA-hydrolysing IgG was demonstrated in patients with systemic lupus erythematosus (Shuster *et al*, 1992), and we demonstrated the presence of thyroglobulin-hydrolysing IgG in a patient with Hashimoto thyroiditis (Li *et al*, 1995). Since then, catalytic antibodies have been associated with a range of pathologies, including multiple myeloma, rheumatoid arthritis, multiple sclerosis, and HIV-related immune thrombocytopenia (Paul *et al*, 1995; Baranovskii *et al*, 2001; Nardi *et al*, 2001; Ponomarenko *et al*, 2002). Catalytic antibodies are also found under physiological conditions in humans. Thus, catalytic antibodies of the IgG and IgM isotypes form part of naturally occurring antibodies (Kalaga *et al*, 1995; Planque *et al*, 2004) and have been suggested to participate in the removal of metabolic waste and protection from bacterial infections (Friboulet *et al*, 1999; Nathan, 2002; Wentworth *et al*, 2002). Interestingly, the presence of IgG with amidolytic activity has been positively associated with survival or positive outcome in severe sepsis (Lacroix-Desmazes *et al*, 2005). Antibodies with hydrolytic properties have been shown to target a large number of self or exogenous antigens; target antigens of particular interest to the present review are proteins involved in blood coagulation – FVIII and FIX (Lacroix-Desmazes *et al*, 1999, 2002; Wootla *et al*, 2008).

FVIII-hydrolysing IgG

In our early work, we demonstrated the presence of IgG with proteolytic activity towards exogenous therapeutic FVIII in inhibitor-positive patients with severe congenital haemophilia A (Lacroix-Desmazes *et al*, 1999). FVIII-hydrolysing IgG were detected in about 50% of the patients included in the study (Lacroix-Desmazes *et al*, 2002). Further, the rates of IgG-mediated FVIII proteolysis measured *in vitro* were positively correlated to the inhibitory activity measured in the plasma of the patients (Lacroix-Desmazes *et al*, 2002), although it is

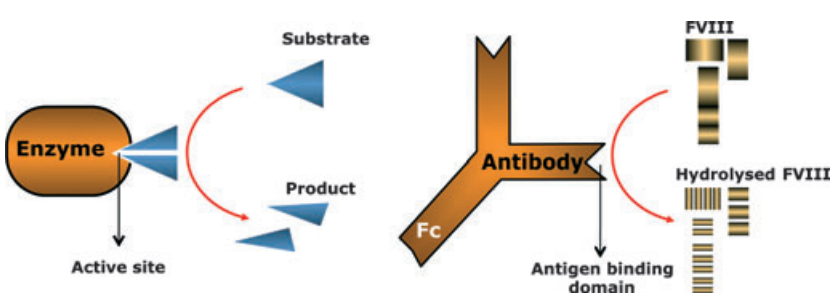

Fig 1. Antigen hydrolysis by catalytic antibodies. The antigen-binding domain of a catalytic antibody mimics the active site of an enzyme. Similar to the enzyme-mediated catalysis of a substrate into its product, catalytic antibodies interact with their antigen (e.g., FVIII) and mediate its hydrolysis.

obvious that the different anti-FVIII IgG in the polyclonal antibody population of a single patient may simultaneously neutralize therapeutic FVIII by several means: classical steric hindrance and proteolysis. Importantly, the proteolysis of FVIII by IgG was demonstrated to lead to FVIII inactivation *in vitro* (Lacroix-Desmazes *et al*, 2006b). The protease activity was similar to that of serine proteases, as more than 80% of the identified cleavage sites on FVIII occurred after a lysine or an arginine (Lacroix-Desmazes *et al*, 2006b).

More recently, we observed that IgG from about 47% of AHA patients also hydrolysed FVIII (Wootla *et al*, 2008). A longitudinal study, performed on four AHA patients to evaluate the relationship between FVIII hydrolysis and FVIII inhibition, suggested a co-evolution of the catalytic activity with the inhibitory titre over time. However, despite the inhibitory potential of FVIII-hydrolysing IgG in AHA patients, no correlation was found between the presence/absence of proteolytic IgG and the clinical parameters of the patients, or the survival status of the patients 1 year after diagnosis. The results of this study did not enable us to attribute the epitope specificity or the type I/II kinetics of FVIII inhibition to the presence of FVIII-hydrolysing IgG.

The nature of the anti-FVIII autoimmune response responsible for AHA differs in several aspects from that of the alloimmune response that develops in patients with congenital haemophilia A; differences in the kinetics of FVIII inactivation and in the capacity to be neutralized by anti-idiotypic antibodies (Lacroix-Desmazes *et al*, 2000) have thus been suggested. The capacity of patients' IgG to hydrolyse FVIII identified one additional distinctive criteria between the two pathological situations. Indeed, IgG-mediated FVIII hydrolysis in congenital haemophilia A is characterized by a higher average hydrolytic rate than that scored in AHA patients (Wootla *et al*, 2009). Further, a significant and positive correlation was found between the hydrolysis of FVIII and of a synthetic chromogenic peptide substrate (proline-phenylalanine-arginine-MCA) in the case of anti-FVIII autoimmune IgG, which was not found with alloantibodies, thus suggesting fine differences in epitope specificity.

Factor IX-hydrolysing IgG

Our initial work on FVIII-hydrolysing IgG in congenital haemophilia A failed to detect hydrolysis of other self-antigens, such as human serum albumin and human recombinant FIX used as controls (Lacroix-Desmazes *et al*, 2002). However, IgG-mediated hydrolysis of FIX was detected, together with FVIII hydrolysis, in one of the patients who survived from severe sepsis (Lacroix-Desmazes *et al*, 2005). No satisfactory explanation could be provided at the time, although it is possible that the combined hydrolysis of FVIII and FIX exerted anti-thrombotic effects and prevented disseminated intravascular coagulation, thus protecting the patient.

Recently, we have found that IgG from some patients with AHA may hydrolyse FIX in addition to FVIII (Wootla *et al*, 2011). The proteolytic activity was a property of the antigen binding site, as it was recovered with the purified $F(ab')_2$ fragments of the patients' IgG. Of note, FIX-hydrolysing IgG were not detected in inhibitor-positive patients with congenital haemophilia B. Taken together, these data suggests that, in contrast to FVIII-hydrolysing IgG that are found in congenital as well as AHA, the spontaneous generation of FIX-hydrolysing IgG requires the endogenous presence of the antigen.

Interestingly, the migration profile of IgG-hydrolysed FIX resembled that of activated FIX (Fig 2). Indeed, N-terminal sequencing of the IgG-mediated FIX degradation products confirmed cleavage of FIX at the arginine 180 residue and we confirmed that IgG-mediated hydrolysis of FIX lead to the activation of the coagulation factor (Fig 3) (Wootla *et al*, 2011). Overall, IgG from 38·5% of the patients included in the cohort were able to activate FIX. IgG-mediated FIX activation tended ($P = 0{\cdot}07$) to be significantly associated with patient survival 12 months following diagnosis. *In vitro* assays showed that the FIX-hydrolysing IgG were able to generate an average 0·3 nmol/l of activated FIX per 24 h. Such a modest amount of activated FIX was able to restore thrombin generation *in vitro*, provided that a basal level of FVIII ≥3% was present in the assay (Wootla *et al*, 2011). Although the measured kinetics of IgG-mediated FIX activation are low when compared to that of physiological FIX-activating enzymes (i.e., activated factor XI), we believe that the low kinetics are compensated by the long half-life and high concentration of IgG in the blood. Moreover, a fraction of the circulating FIX upon IgG-mediated activation,

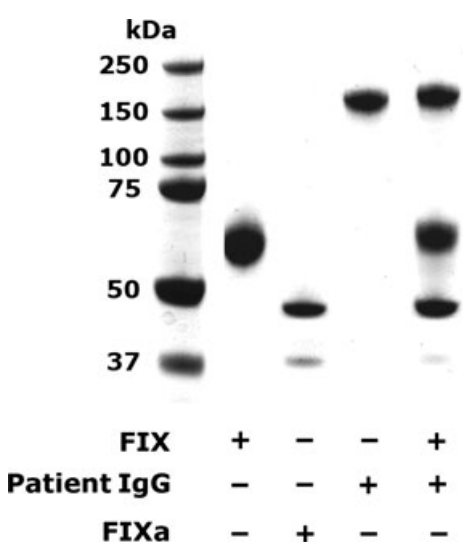

Fig 2. Hydrolysis of factor IX by purified IgG from a patient with AHA. Human recombinant factor IX (FIX), human recombinant activated factor IX (FIXa), IgG purified from the plasma of a patient with AHA, and FIX incubated in the presence of the patient's IgG were subjected to 4–12% sodium dodecyl sulphate polyacrylamide gel electrophoresis. Proteins were stained by colloidal Coomassie blue. Incubation of FIX with patient's IgG resulted in the generation of a band corresponding to the size of FIXa.

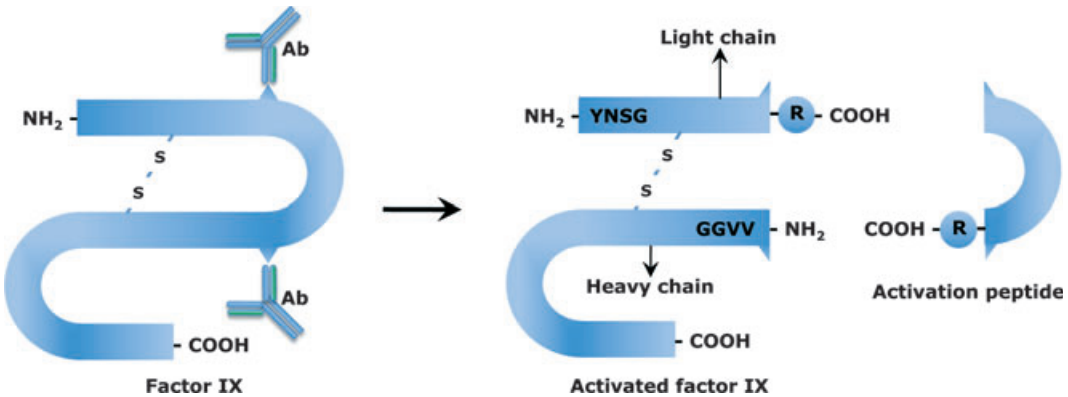

Fig 3. Activation of factor IX by purified IgG from a patient with AHA. IgG-mediated FIX degradation products, as seen in Fig 2, were subjected to Edman degradation. Two amino acid sequences were identified that correspond to the N-terminus of factor IX (YNSG) and N-terminus of the heavy chain of factor IX (VVGG). IgG antibodies (Ab) from AHA patients specifically hydrolysed factor IX at the carboxy-terminal of the arginine residues (R-COOH), resulting in the release of the activation peptide. The release of the activation peptide yielded activated FIX.

may feed in the coagulation cascade and compensate for the loss of FVIII activity in AHA patients.

Conclusion

AHA is a serious disease with a high mortality rate. The reasons for the elevated mortality are mostly related to the old age of the patients, to particular underlying pathologies (e.g., cancer) or to the difficulty to re-establish haemostasis and immune homeostasis in the patients. The data accumulated in the recent past emphasizes the association of certain gene polymorphisms with AHA development, as well as the importance of autoreactive $CD4^+$ T lymphocytes in the generation of the FVIII inhibitors. The observations however do not open obvious avenues towards better management of the patients. An original finding in the last year is the observation of FIX-hydrolysing IgG that activates the molecule in about a third of AHA patients. *In vitro* data suggest that the generation of activated FIX by IgG re-establishes thrombin generation in a situation where some residual FVIII activity is present. To date, haemorrhages in patients with AHA are treated with FVIII, activated factor VII, or activated prothrombin complexes, all of which are hampered by short half-life and/or risk for thromboembolic complications. Our recent findings raise the issue of the therapeutic relevance of the passive administration of FIX-activating IgG. Proteolytic antibodies would advantageously combine properties of enzymes, which is 'turnover', and properties of antibodies, that is long half-life. Moreover, FIX-activating antibodies would pose low risk for thrombotic complications because of their low catalytic rates of FIX activation.

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Conclusion:

Antibodies are versatile molecules known to play a plethora of functions. Indeed it is the inherent diversity installed in the immunoglobulin repertoire that enables antibodies to perform several functions. Our present knowledge on antibody functions have extend from binding to single cognate antigen, to polyreactivity and even catalytic activity.

Although it has been 25 years since the development of the first catalytic antibody, our knowledge on their physiopathological role is still rudimentary. During my PhD, I tried to understand some of the basic questions regarding catalytic antibodies. Intially thought to be deleterious in disease, they are now considered to be molecules involved in maintaining immune homeostasis. Our study on acquired hemophilia and renal transplant provides newer insights into the beneficial aspects of catalytic antibodies. We observed a novel function of FIX activation by patients' IgG, which showed a tendency towards better survival of the patients. We believe that FIX activating antibodies can be a good therapeutic tool for treating patients with acquired hemophilia. In addition our study on patients with renal transplant provided an insight on the evolution of catalytic antibodies during the course of the disease. The results of this study will have implication in future in the utility of catalytic antibodies as a predictive tool for chronic allograft nephropathy.

Finally our results on polyreactivity have provided greater insight on the mechanism of heme-induced polyreactivity. In perspective we wish to revisit the phenomenon by re-cloning the variable region genes in an IgM cloning vector.