IDENTIFICATION OF PROTEIN'S COMPLEMENTARY TO THE AUTOANTIGEN PROTEINASE 3 AND THEIR INVOLVEMENT IN THE PATHOGENESIS OF AUTOIMMUNE DISEASE

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ABSTRACT

David James Bautz: Identification of Protein's Complementary to the Autoantigen Proteinase 3 and Their Involvement in the Pathogenesis of Autoimmune Disease

(Under the direction of Ronald Falk, M.D., Gloria Preston, Ph.D. and Alex Tropsha, Ph.D.)

Previous work by our research group showed that PR3-ANCA patients had an antibody response to a recombinant complementary-PR3 protein encoded by the antisense strand of the PR3 mRNA. To follow up on this work, we sought to determine whether the patients also had a T cell response to this recombinant complementary-PR3 protein and whether a protein reactive with those antibodies could be identified *in vivo*.

Chapter 2 of the dissertation describes the identification of $CD4^+$ T_H1 cells that proliferate in response to a complementary-PR3 peptide. This proliferation was seen by both a CFSE assay as well as by interferon-γ production in an ELISPOT assay. Those patients who had a T cell response to complementary-PR3 peptide also had antibodies to the complementary-PR3 protein.

We next sought to determine if complementary-PR3 proteins could be identified from patient plasmapheresis material. Chapter 3 of this dissertation describes the identification of two complementary-PR3 proteins, human plasminogen and Protein F, a protein from pseudomonas. These proteins reacted with an antibody raised to a peptide encoded by the antisense RNA of the PR3 gene. As complementary proteins are known to interact,

plasminogen was shown to be a substrate of PR3, indicative of interaction between the two proteins. Lastly, the anti-complementary PR3 antibodies also bound to normal human leukocytes, cells that are known to bind plasminogen on their surface.

Chapter 4 describes the identification of anti-plasminogen autoantibodies in PR3-ANCA positive patients. These antibodies were purified using a complementary-PR3 peptide column, indicating that the anti-cPR3 and anti-plasminogen antibodies are the same. The anti-plasminogen antibodies bound a surface-exposed loop on plasminogen's catalytic domain. Two *in vitro* assays confirmed the antibodies affect on plasminogen activity. Serological screening of sera indicated that the anti-plasminogen autoantibodies were more prevalent in those PR3-ANCA patients with a clinical history of venous thrombotic events.

By designing an experimental approach that considered protein complementarity, a previously unknown autoantigen and its pathogenic autoantibodies were identified. Consideration of complementary proteins can be used to discover other, and perhaps proximal, autoantigens and autoantibodies in other autoimmune diseases.

For my beautiful wife Kari, whose unending support and sacrifice made this possible.

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The work presented here would not have been possible without the aid and assistance of a host of individuals. I would first and foremost like to thank my wife, Kari, for agreeing to the idea of moving to North Carolina and me taking a 60% pay cut to come back to school. I'm not sure many people would agree to that, but I am very thankful that she did. I'm grateful to my mother for her continuous love, support and encouragement. She's always been eager to hear about what I've been up to in the lab. I would like to thank Dr. Barry Lentz for admitting me to the Biophysics Program, which allowed me to investigate a wide array of laboratories to perform my dissertation work in. I never would have ended up working on this project if Dr. Alex Tropsha had not set up a meeting to introduce me to Drs. Ron Falk and Gloria Preston, and for that I am very grateful. It has been a real pleasure to work with Drs. Falk and Preston due to their outstanding commitment to quality science but also because of the laid back and down to earth nature which they go about it. I can't imagine too many other graduate students having the opportunities to work with such a diverse and talented group of scientists as are assembled here at the UNC Kidney Center, and I consider myself extremely fortunate to have been given this opportunity. I'd also like to thank the other members of my dissertation committee, Drs. Brian Kuhlman, Stephen Chaney, and Christoph Borchers for asking probing questions and offering insightful advice about the project. I'd like to thank each of the following individuals for helping me out in their own unique ways:

As the other graduate student in the lab, Dr. Josh Astern could appreciate what life was like as a grad student and it was nice to have someone in the lab going through the same experiences. Josh was also quite adept at providing comic relief to lighten the mood of the lab and there were certainly times when that was needed and appreciated.

Not having to worry about ordering supplies allowed me time to work on my various projects, and for that I am indebted to Nirmal Khandoobai since he took care of all supply ordering and restocking, along with so many other laboratory duties. I also thank Nirmal for making my trip to Cancun for the 2007 ANCA meeting as fun as possible.

I'd like to thank Julie Hamra for identifying patients coming into the clinic, collecting all of our patient samples, and relaying pertinent patient information.

The data generated from this project was made all the more important because of the detailed patient information collated by Dr. Sophia Lionaki. I am very grateful to her for going through stacks and stacks of patient charts, calculating untold numbers of BVAS scores, and preparing tables on patient data

This project involved doing untold number of ELISA's, and I'd like to thank Dr. JJ Yang for all of her help with those. In addition, JJ was adept at essentially every assay done in this lab and was always willing and eager to train and I thank her for taking time to teach me all those various laboratory techniques.

I'm indebted to Dr. Peter Hewins for always being willing to lend a hand in any way, shape or form. I consider myself very fortunate to have been able to work with such an intelligent scientist.

Having enough recombinant protein available for all the assays performed in these experiments was crucial, and I'd like to thank Frances Belmonte and Therese Tulley for their aid and assistance in making sure there was enough protein available at all times.

I'd like to thank both Dr. Susan Hogan and Hyunsook Chin for their work on the statistical analysis.

Epitope mapping of the anti-plasminogen antibodies was an important piece of data generated during this project, and I'd like to thank Dr. Viorel Mocanu and Dr. Carol Parker for their assistance in epitope mapping of antibody samples using mass spectrometry. I was especially appreciative of their willingness to explain how the epitope mapping worked and to let me sit in on some of those experiments.

Assaying antibodies directed against plasminogen was something that our research group had never done before, thus I am indebted to Dr. Alisa Wolberg for providing ideas for assays to test activity of anti-plasminogen autoantibodies and for training me in those asssays.

Lastly, I'd like to thank my daughter Kira. She has brought more joy to my life than I thought possible and watching her grow up is the greatest biology experiment I'll ever be a part of.

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ABBREVIATIONS

AChR	Acetylcholine receptor
ACTH	Adenocorticotropin
AMA	Anti-mitochondrial antibody
ANCA	Anti-neutrophil cytoplasmic autoantibody
AP	Alkaline phosphatase
APGN	Anti-plasminogen autoantibodies
AZA	Azathioprine
Bis-Q	Trans-3,3'-bis[alpha-(trimethylammonia)-methyl]azobenzene
BSA	Bovine serum albumin
BVAS	Birmingham vasculitis activity score
Ca^{2+}	Calcium ion
c-ANCA	ANCA producing a cytoplasmic staining pattern
CDI	Cell division index
cDNA	complementary DNA
CMV	Cytomegalovirus
CNBr	Cyanogen bromide
ConA	Concavalin A
cPR3	Complementary PR3
$cPR3^{(105\text{-}201)}$	Complementary PR3 protein corresponding to PR3 residues 105-201
$cPR3^{(138\text{-}169)}$	Complementary PR3 peptide corresponding to PR3 residues 138-169

CsA	Cycolsporine
CSFE	Carboxy-fluorescein diacetate succinimidyl ester
CSS	Churg-Strauss syndrome
CV	Column volumes
CYC	Cyclophosphamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
DVT	Deep vein thrombosis
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
FITC	Fluorescein isothiocyanate
FPLC	Fast performance liquid chromatography
FSH	Human follicle stimulating hormone
g	Force of gravity
GAS	Group A streptococcus
GC	Glucocorticoids
HBS	Hepes buffered saline
HCl	Hydrochloric acid
HEK 293	Human embryonic kidney cell line
HEPES	4-(2-hydroxymethyl)-1-peperazineethanesulfonic acid

HI	Heat inactivated
HRP	Horseradish peroxidase
HTCA	Complementary ACTH peptide
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IgY	Immunoglobulin Y
IRB	Institutional review board
ITP	Idiopathic thrombocytopenia purpura
IVIg	Intravenous immunoglobulin
kDa	Kilodalton
KLH	Keyhole limpet hemocyanin
La/SSB	Ribonucleoprotein
MALDI-TOF	Matrix assisted laser desorpton/ionization-time of flight
MG	Myasthenia gravis
MPA	Microscopic polyangitis
MMF	Mycophenolate mofetil
MPO	Myeloperoxidase
MRT	Molecular recognition theory
MS	Mass spectrometry
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaN_3	Sodium Azide
OD	Optical density

p-ANCA	ANCA producing a perinuclear staining pattern
PBS	Phosphate buffered saline
PDB	Protein data bank
PE	Phycoerythrin
pgp 1b	Platelet glycoprotein 1b
PLEX	Plasmapheresis fluid
PMA	Phorbol 12-myristole 13-acetate
PR3	Proteinase 3
PRTN3	Gene encoding PR3
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
SVV	Small vessel vasculitis
tPA	Tissue-type plasminogen activator
TPCK	Tosyl phenylalanyl chloromethyl ketone
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
TSH	Thyroid stimulating hormone
uPA	Urokinase-type plasminogen activator
VTE	Venous thrombotic event
VWF	von Willebrand factor
WG	Wegener's granulomatosis

SYMBOLS

β	Beta
©	Copyright
°	Degrees
γ	Gamma
μ	Micro
±	Plus or minus

CHAPTER I

PROLOGUE

The work described in this dissertation touches upon one of the most complex issues in the biological sciences; how and why does the immune system, which is designed to protect the individual from pathogenic invaders, decide that the host is in fact the enemy? This question is the basis for studying autoimmune disease, and until it can be answered with confidence it will remain incredibly difficult to discover better cures and treatments for patients suffering from these debilitating diseases.

Before describing the rationale for pursuing this project and the data subsequently generated, I will provide a discussion of ideas and theories that were combined in this research. These theories may at first appear to be unrelated; however it will become apparent that as a result of not compartmentalizing theories and ideas a new model for discovering autoantigens and autoantibodies was discovered. The dissertation will begin with an overview of the literature from which the major themes were generated that influenced the design and interpretation of the studies described.

AUTOIMMUNE DISEASE

Discerning the underlying cause of human disease is crucial to develop better and more effective treatments for a leading cause of suffering and mortality, autoimmune disease. While much effort has been put into the study of heart disease, cancer, and stroke, scientists are beginning to focus more on a loose-knit group of poorly understood diseases mediated by the immune system.

Autoimmune diseases are characterized by the apparent unregulated attack by the immune system on self tissue. There are over 80 autoimmune diseases currently identified that affect 14-22 million Americans [1]. They include common conditions such as Grave's disease (1,150 per 100,000 individuals) as well as less frequent ailments such as Myasthenia gravis (0.4 per 100,000 individuals) [2]. Autoimmune diseases strike people of all ages, ethnicities, and backgrounds, however they typically affect women more frequently than men (>75% of those affected are women) for reasons that remain unclear [3].

Criteria have been introduced to help distinguish autoimmune diseases at three different levels: direct, indirect and circumstantial [4]. Direct evidence for an autoimmune disease requires transmissibility of the characteristic wounds from either human to human or human to animal. Indirect evidence requires an animal model that re-creates the disease. Circumstantial evidence of autoimmune disease relies on "markers", such as presence of high levels of autoantibodies in serum and/or deposition of antibody/antigen complexes in the affected organ.

Autoimmune diseases are classified as either organ-specific or systemic. Grave's disease is an example of an organ-specific disease as it is characterized by an autoantibody response against the thyroid [5]. Examples of systemic autoimmune diseases include

Wegener's granulomatosis (WG), microscopic polyangiitis (MPA), and Churg-Strauss syndrome (CSS), all of which are characterized by vasculitis affecting multiple organ systems, typically the small-sized vessels of the lungs and/or kidneys, as well as the presence of autoantibodies directed against proteins found in neutrophils [6].

SMALL-VESSEL VASCULITIS

Vasculitis is the term given to the group of uncommon diseases that result in inflammation of the blood vessels. Vessels of any type and of any organ can be affected. Vasculitis has many causes, one of which is through an autoimmune response. One way of categorizing different vasculitides is based on the size of the blood vessels they affect. Small-vessel vasculitides are defined as vasculitis that affects vessels smaller than the arteries, such as the arterioles and capillaries [7]. These diseases strike individuals of all ages, however they typically affect people in their 50's and 60's with the occurrence of these diseases similar between men and women [8]. The disease is quite rare, as it only affects approximately 1 in 100,000 people per year in Sweden [9] and 2 in 100,000 in the United Kingdom [10]. The aforementioned small-vessel vasculitides (WG, MPA, CSS) were determined to be closely related as early as the 1950's [11]. This was confirmed by the discovery of circulating autoantibodies directed against constituents of the neutrophil and monocyte in patients suffering from these diseases. These autoantibodies, termed ANCA (Anti-Neutrophil Cytoplasmic Autoantibodies), are present in approximately 90% of patients suffering from WG and MPA [12].

WEGENER'S GRANULOMATOSIS

The condition of WG was first reported by Klinger in 1931 [13]. A more detailed description of the disease was later given by Friedrich Wegener [14]. The term "Wegener's granulomatosis" was not introduced until 1954 by Godman and Churg [11]. Patients suffering from WG typically have inflammation of the small- and medium-sized blood vessels along with a number of different symptoms that affect various organ systems, however over 90% of WG patients have upper or lower respiratory tract disease [15]. Target organs include the upper airway (typically crusting and bleeding in the nasal passages), the lungs (pulmonary nodules and hemoptysis), and the kidneys (rapidly progressing segmental necrotizing glomerulonephritis) [16]. The American College of Rheumatology published acceptance criteria for WG [17], which was followed up by the Chapel Hill Consensus Conference on nomenclature of systemic vasculitis, and concluded that "the term 'Wegener's Granulomatosis' is restricted to patients with granulomatous inflammation" [18].

Treatment needs to commence quickly after diagnosis, as this disease leads to organ failure and potentially death without intervention. The treatment of WG and other SVV occurs in three stages: induction of remission, maintaining remission, and treatment of relapse [19]. Patients who have aggressive disease are typically treated with intravenous corticosteroids and cyclophosphamide [7]. Combined therapy with those two drugs typically results in improvement of disease in 90 percent of patients and complete remission in 90 percent [20]. The use of steroids and immuno-suppressive therapy has helped to make this a more manageable ailment, however these treatments are very caustic to the patient and could potentially lead to serious infections [7, 21].

ANCA

A feature that distinguishes WG, MPA and CSS from other small-vessel vasculitides is the presence of circulating ANCA. ANCA, first described by Davies et al. in 1982 [22] and in WG in 1985 [23], are typically present in 85-95% of patients with small-vessel vasculitides [24]. An indirect immunofluorescence test is used to verify the presence of ANCA. This test involves staining ethanol-fixed neutrophils with patient sera. The antibodies are classified on the basis of their neutrophil staining pattern (Figure 1.1); either c-ANCA (cytoplasmic ANCA) or p-ANCA (perinuclear ANCA). The most common c-ANCA antigen is proteinase 3 (PR3), primarily associated with WG [25, 26], while the most common p-ANCA antigen is myeloperoxidase (MPO), which is largely associated with MPA [27]. An atypical staining pattern can also be seen with the target antigens bacterial/permeability increasing protein, elastase and catalase [28], however they are not associated with vasculitic autoimmune diseases. ANCA are present to a lesser degree in other autoimmune diseases such as Sjogren's syndrome (target antigen is lactoferrin) [29] and ulcerative colitis (target antigen is cathepsin G) [30]. When immunofluorescence staining is combined with ELISA testing the specificity for ANCA small-vessel vasculitis (SVV) is 99% and sensitivity for WG is 73% [31].

An interesting feature of small-vessel vasculitides is the lack of immune deposits along the vasculature [32]. This lack of ANCA antibody deposits has made proving ANCA causation difficult. Strong data supporting a role of ANCA pathogenesis have come from numerous *in vitro* and *in vivo* studies. It was shown that ANCA activate neutrophils *in vitro*, causing the release of reactive oxygen species and primary granule contents [33]. Van Rossum et al. showed that only those neutrophils that express PR3 on their surface were able

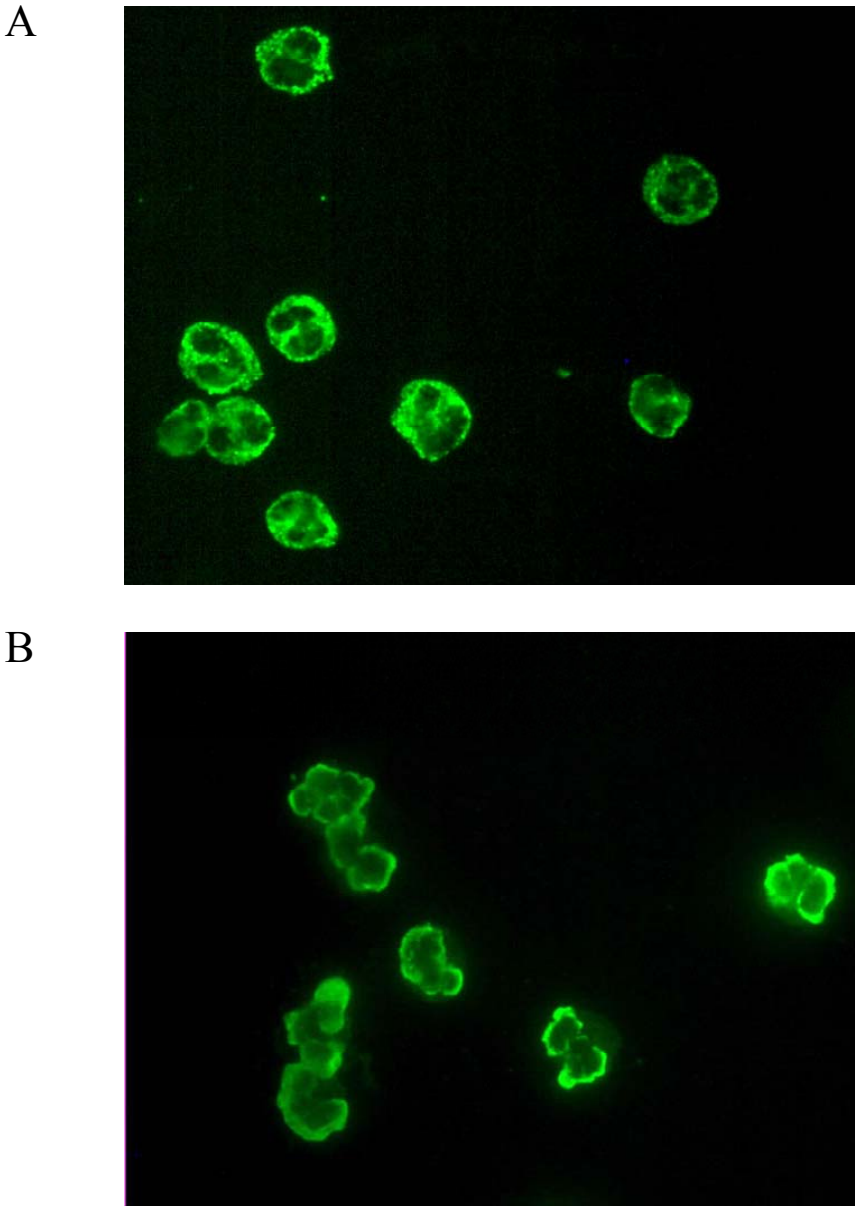

Figure 1.1. ANCA Staining of Human Neutrophils.
Human neutrophils are fixed with ethanol before staining with patient sera and a FITC-labeled secondary antibody. (A) cANCA staining pattern, showing a grainy, diffuse staining of the cytoplasm. The most common autoantigen resulting in a cANCA staining pattern is proteinase 3. (B) pANCA staining pattern showing a perinuclear staining with little staining of the cytoplasm. The most common autoantigen resulting in a pANCA staining pattern is myeloperoxidase.

to bind PR3-ANCA from Wegener's patients in a dose-dependent fashion [34]. There is now evidence from experimental animal models that ANCA are pathogenic and can cause disease [35]. Xiao et al. injected MPO into MPO knockout mice and then transferred splenocytes from these animals into mice deficient in B and T lymphocytes. Mice that received anti-MPO splenocytes developed "severe necrotizing and crescentic glomerulonephritis, granulomatous inflammation, and systemic necrotizing vasculitis" [35]. While MPO-ANCA have been shown to be pathogenic, there is still no animal model to prove the same relationship with PR3-ANCA.

PROTEINASE 3

Proteinase 3 (PR3) was first described in 1975 by Dewald et al. [36] and further characterized by Kao et al. during research into whether granulocyte proteins other than elastase could be involved in emphysema formation in humans [37]. PR3, along with elastase and azurocidin, is part of a family of serine proteases whose genes are located in a cluster on chromosome 19 that are synchronously expressed in the early stages of granulopoiesis in the bone marrow [38]. PR3's function is associated with a number of different cellular activities, including inhibition of NADPH oxidase [39], apoptosis [40], and myeloid differentiation [41]. PR3 is also expressed on the surface of neutrophils in the enzymatically active form, however, unlike the granule localized form, it is resistant to inhibition [42].

PR3 was identified as a major ANCA target autoantigen at approximately the same time by a number of different research groups [25, 43-45]. It has been shown that expression of both PR3 and MPO message is upregulated in ANCA patient neutrophils [46]. In

addition, the presence of PR3 on the surface of neutrophils has been correlated with disease activity [47]. Taken together, these data suggest that there is amble PR3 antigen available for interaction with ANCA to cause disease in patients.

There appear to be multiple epitopes recognized by ANCA, and most of these are conformational epitopes [48]. Since there is little consensus on what epitopes are important on PR3, our research group became interested in epitope mapping PR3-ANCA. To do this, a random peptide library in bacteria was produced using blunt-ended fragments of the PR3 cDNA. Since the fragments were blunt-ended, they could insert into the vector either in the correct orientation or in the "flipped" orientation, generating PR3 peptides or peptides off the antisense strand of the gene. The expressed peptide fragments were then tested for reactivity with PR3-ANCA. The results of these experiments showed that a subset of PR3-ANCA patients had antibodies not only to PR3, but also to a complementary protein expressed off the antisense strand of the PR3 gene, which is referred to as complementary PR3, or *cPR3* [49]. These unexpected findings led to a series of experiments and ultimately to the development of a new theory of autoimmune disease formation, the theory of autoantigen complementarity.

COMPLEMENTARY PROTEINS

Complementary proteins are the protein equivalent to complementary nucleotide sequences, that is, they are proteins or peptides expressed in-frame off complementary DNA or RNA strands. The idea of complementary proteins was first introduced by Mekler in 1969 [50]. He recorded that the active, dimeric form of ribonuclease A was held together by specific amino acid interactions. He found that reading the complementary RNA encoding

one of the interacting polypeptides in the 5' -> 3' direction yielded the amino acid sequence of the other interacting chain. Little work was done with regard to complementary proteins until Mekler's ideas were reinvented by Biro [51] and Blalock/Smith [52] in the 1980's. The basic theory put forth by this group of scientists is that peptides and proteins that selectively bind to one another are encoded by in-frame codons on complementary DNA/RNA strands. This idea has also been given the term "sense-antisense" that describes the proteins being encoded by sense and antisense nucleotide strands. Due to the degeneracy of the genetic code, there are multiple antisense partners for most amino acids (Table 1.1).

Based on the Mekler definition of complementary amino acids, a hydrophobic amino acid is always encoded opposite a hydrophilic partner [50, 52]. Thus, theoretically, for complementary proteins to interact it would require hydrophobic and hydrophilic amino acids to interact. While intuitively this seems rather unappealing, there is some evidence to support this arrangement. For example, leucine and lysine have been shown to interact spontaneously in solution [53]. It has also been suggested that binding between complementary proteins could be caused by the interactions between the hydrophobic side chains and the hydrocarbon backbone supporting the polar residues of their complementary partner [54, 55]. A model for the side-chain packing of glutamic acid and leucine has also been reported [56].

While there is no agreed upon explanation for why complementary proteins interact, there are numerous examples of interactions in the literature [57]. Jones was the first to perform experiments with complementary peptides [58]. He designed peptides to be antisense to the C-terminal portion of gastrin and all peptides produced, except for one, were

Table 1.1. Sense and Antisense Codon Table.

Amino Acid		Codon 5' -> 3'	AS* Codon 5' -> 3'	AS Amino Acid		Amino Acid		Codon 5' -> 3'	AS Codon 5' -> 3'	AS Amino Acid	
Alanine	A	GCT	AGC	Serine	S	Leucine	L	TTA	TAA	Stop	-
		GCC	GGC	Glycine	G			TTG	CAA	Glutamine	Q
		GCA	TGC	Cysteine	C			CTT	AAG	Lysine	K
		GCG	CGC	Arginine	R			CTC	GAG	Glutamate	E
								CTA	TAG	Stop	-
								CTG	CAG	Glutamine	Q
Arginine	R	CGT	ACG	Threonine	T	Lysine	K	AAA	TTT	Phenylalanine	F
		CGC	GCG	Alanine	A			AAG	CTT	Leucine	L
		CGA	TCG	Serine	S						
		CGG	CCG	Proline	P	Methionine	M	ATG	CAT	Histidine	H
		AGA	TCT	Serine	S						
		AGG	CCT	Proline	P	Phenylalanine	F	TTT	AAA	Lysine	K
								TTC	GAA	Glutamate	E
Asparagine	N	AAT	ATT	Isoleucine	I	Proline	P	CCT	AGG	Arginine	R
		AAC	GTT	Valine	V			CCC	GGG	Glycine	G
								CCA	TGG	Tryptophan	W
Aspartate	D	GAT	ATC	Isoleucine	I			CCG	CGG	Arginine	R
		GAC	GTC	Valine	V						
Cysteine	C	TGT	ACA	Threonine	T	Serine	S	AGT	ACT	Threonine	T
		TGC	GCA	Alanine	A			AGC	GCT	Alanine	A
								TCT	AGA	Arginine	R
								TCC	GGA	Glycine	G
Glutamic Acid	E	GAA	TTC	Phenylalanine	F			TCA	TGA	Stop	-
		GAG	CTC	Leucine	L			TCG	CGA	Arginine	R
Glutamine	Q	CAA	TTG	Leucine	L	Threonine	T	ACT	AGT	Serine	S
		CAG	CTG	Leucine	L			ACC	GGT	Glycine	G
								ACA	TGT	Cysteine	C
								ACG	CGT	Arginine	R
Glycine	G	GGT	ACC	Threonine	T	Tryptophan	W	TGG	CCA	Proline	P
		GGC	GCC	Alanine	A						
		GGA	TCC	Serine	S	Tyrosine	Y	TAT	ATA	Isoleucine	I
		GGG	CCC	Proline	P			TAC	GTA	Valine	V
Histidine	H	CAT	ATG	Methionine	M						
		CAC	GTG	Valine	V	Valine	V	GTT	AAC	Asparagine	N
								GTC	GAC	Aspartate	D
Isoleucine	I	ATT	AAT	Asparagine	N			GTA	TAC	Tyrosine	Y
		ATC	GAT	Aspartate	D			GTG	CAC	Histidine	H
		ATA	TAT	Tyrosine	Y						

* AS = antisense

biologically active. Bost et al. produced a complementary peptide to adrenocorticotropin hormone (ACTH) by reading the antisense nucleotide sequence in-frame in the 5'->3' direction. This peptide, which they termed HTCA, bound to ACTH with high affinity [59]. Ghiso et al. suggested that the interaction between cystatin C, a cysteine proteinase inhibitor, and complement C4 is mediated by segments coded by complementary DNA sequences of the two proteins [60]. Much of the work thus far involving complementary peptides has been with receptor-ligand interactions, for example with angiotensin II [61] and human follicle stimulating hormone (FSH) [62]. However, it should be noted that there is a lot of controversy surrounding the validity of complementary protein interactions and much of the data generated thus far has been debatable and contradictory [63, 64].

Since the idea of hydrophobic and hydrophilic amino acids interacting is so intellectually unappealing, Blalock hypothesized that complementary peptides would assume complementary shapes due to the "inverse forces" working on each peptide due to their inverted hydropathic profile. He suggested that it is hydropathic complementarity that is driving the specific interactions between complementary peptides, not specific interactions between amino acids with opposing hydropathies. His ideas have been formally put forth as the molecular recognition theory (MRT) [65]. Furthermore, Blalock and coworkers suggested that the idiotypic network theory, said to be composed of complementary cellular interactions that regulate the immune system, results from complementary shapes brought about by complementary hydropathic profiles [66].

IDIOTYPIC NETWORK THEORY

Neils Jerne theorized that the immune system is composed of a large network of interacting antibodies and receptors [67]. He postulated that an antibody molecule contains a paratope, or antibody combining site, that itself is composed of idiotopes, or epitopes displayed by the variable regions of antibodies. Thus, the paratope and idiotopes are expressed together and are dependent upon the primary sequence of the antibody variable region. It is these idiotopes, a group of which is called an idiotype, that are recognized by other antibodies in the immune system.

If an antigen is introduced to the immune system, there will be a response by a group of antibodies. The antigen combining sites on these antibodies will contain unique sequences that are unfamiliar to the immune system, thus an immune response will be generated against these idiotypes. Jerne theorized that these anti-idiotypic antibodies will bear the "internal image" of the original antigen. He also believed that the idiotypic network would play a role in suppressing an antibody response that in turn would lead the entire system back to a state of equilibrium. His idea's were confirmed in a number of experiments by his research group that showed anti-idiotypic antibodies were produced in rabbits injected with immunoglobulin [68].

Jerne's work has also been supported by a number of studies by other groups examining the nature of idiotypic interactions. Braden et. al. produced a crystal structure of the monoclonal antibody D1.3 (that binds chicken egg-white lysozyme) binding to a monoclonal anti-idiotypic antibody E5.2 [69]. They showed that E5.2 and D1.3 had approximately the same topological and binding group mimicry as between D1.3 and lysozyme, supporting the idea that E5.2 carried the "internal image" of lysozyme. Mimicry

of receptor/ligand interactions by anti-idiotypic antibodies has also been extensively reported [70].

IDIOTYPES AND AUTOIMMUNITY

Idiotypic antibody interactions have also been reported in the study of a number of autoimmune diseases. Shechter et al. found that mice injected with insulin developed antibodies not only to insulin, but to insulin receptor [71]. These anti-insulin receptor antibodies bound to insulin receptor, displaced already bound insulin, and mimicked the actions of insulin in stimulating the oxidation of glucose and inhibiting lipolysis. The binding of anti-insulin receptor antibodies to insulin receptor could be blocked by anti-insulin antibodies suggesting the two antibodies formed an idiotypic pair. To characterize these antibodies further, the group produced chemically altered insulin that did not bind to insulin receptor. This altered insulin produced high levels of antibody, however no anti-idiotypic antibody that bound to insulin receptor was produced. The group concluded that the epitope responsible for the specific idiotypic network was most likely the part of insulin recognized by the insulin receptor [72].

Anti-idiotypic antibodies were reported to be responsible for the therapeutic affects of intravenous immunoglobulin (IVIg) in the treatment of patients with ANCA vasculitis [73]. To follow up on this observation, Jayne et al. searched for anti-idiotypic antibodies in patients with MPO-ANCA. They found a reciprocal relationship between MPO-ANCA and anti-MPO-ANCA, i.e. when the MPO-ANCA level was high, the anti-idiotypic antibody level was low and vice versa [74]. The same group recorded a similar finding with regard to patients suffering from primary biliary cirrhosis. These patients typically have anti-

mitochondrial antibodies (AMA). When a monoclonal antibody was raised to the E2 complex of pyruvate dehydrogenase complex (the target antigen of AMA), it was able to bind anti-idiotypic antibodies from sera. The specificity of these anti-idiotypic antibodies was shown by their ability to inhibit binding of AMA to mitochondria but not other autoantibodies to their respective autoantigens [75]. The group did not investigate whether these autoantibodies were involved in control of AMA.

The study of myasthenia gravis (MG), an autoimmune disease characterized by antibodies to acetylcholine receptor (AChR), has resulted in a further understanding of the idiotypic network and how it relates to autoimmune disease. Erlanger, et al. produced a highly potent agonist to acetylcholine receptor, BisQ, and utilized this compound in the study of antibodies to AChR. They produced antibodies to BisQ and immunized rabbits with these anti-BisQ antibodies, the hypothesis being that the anti-anti-BisQ antibodies would mimic BisQ and bind to AChR and thus potentially cause disease. Out of 5 rabbits immunized with anti-BisQ antibodies, 4 of them showed signs of MG [76]. In addition to showing signs of disease, the rabbits also exhibited anti-AChR antibodies by ELISA. This work was followed up by immunization of mice with BisQ to allow the formation of anti-anti-BisQ antibodies to occur through the intact idiotypic network. The researchers made hybridoma cell lines and screened for anti-anti-BisQ antibodies using their rabbit anti-BisQ antibody. They found a very substantial "auto-anti-idioytpic" response (i.e. a high level of anti-anti-BisQ antibodies) [77]. At around the same time, and by accident, a second group identified anti-idiotypic antibodies to anti-AChR antibodies while working on an anti-AChR ELISA in humans with MG [78].

Erlanger et al. utilized the same strategy for the identification of anti-idiotypic antibodies in a patient with Grave's disease, an ailment characterized by autoantibodies directed against thyroid-stimulating hormone (TSH) receptor. Immunization of mice with both bovine and human TSH resulted in the production of hybridomas that produced anti-TSH antibodies as well as anti-idiotypic antibodies. The researchers then used one of the monoclonal anti-TSH antibodies (LE-4) to make an affinity column and passed human plasmapheresis fluid from a Grave's disease patient over the column. The eluted antibody inhibited the binding of LE-4 to TSH and also bound to the TSH receptor, thus showing the presence of anti-idiotypic antibodies in a human patient [79].

Mice with experimental systemic lupus erythamatosus (SLE) have anti-double stranded (ds) DNA antibodies. Since the idiotypic network has been hypothesized to control autoantibody reactivity, it was hypothesized that anti-anti-dsDNA antibodies would alleviate symptoms in SLE mice. To test this, Shoenfeld et al. affinity purified anti-anti-dsDNA antibodies from IVIg (IVIg-ID) using an affinity column composed of anti-dsDNA antibodies isolated from 55 patients with active SLE. They then treated mice with normal IVIg and IVIg-ID before and after the mice developed anti-dsDNA antibodies. The mice treated with IVIg-ID had significantly lower proteinurea, a longer survival time and a decrease in anti-dsDNA antibodies [80].

COMPLEMENTARY PROTEINS AND THE IDIOTYPIC NETWORK

Since Jerne's network theory presupposes a network of receptors and antibody combining sites with "complementary" shapes, and since peptides encoded by sense-antisense strands of DNA were hypothesized to have complementary conformations, Smith et

al. tested whether antibodies to complementary peptides would interact in an idiotypic fashion [66]. To test this, they injected a rabbit with ACTH and a second rabbit with HTCA. They then isolated total IgG from both rabbits and showed that antibodies specific to ACTH from one rabbit bound to antibodies to HTCA from the other rabbit through their combining sites; they were an idiotypic pair. This work was followed up by the same group using a pair of arbitrary complementary peptides [81]. Figure 1.2 gives a graphical representation of the relationship between complementary proteins and the idiotypic network.

If anti-idiotypic antibodies can be derived from complementary peptides, one potential application for complementary peptides could be their use in treating autoimmune disease. It follows that if anti-idiotypic antibodies could be induced, above the level already seen naturally in the disease, they could potentially block the action of harmful autoantibodies. This has been shown to be the case in a rat model of MG, where both a monoclonal antibody to an antisense peptide (corresponding to the autoantibody binding site on acetylcholine receptor) and an antisense peptide itself have alleviated symptoms [82, 83].

The application of complementary peptides and the existence of an intact idiotypic network in human autoimmune disease was shown by Routsias et al. in the study of Sjogren's syndrome and SLE [84]. Autoantibodies to La/SSB (ribonucleoproteins) are typically found in patients suffering from these diseases. The group was investigating whether patients had anti-idiotypic antibodies to antibodies specific for known determinants on La/SSB. They produced a complementary peptide to the known epitope on La/SSB using the deduced sequence from the non-coding strand of the La/SSB gene and demonstrated that patients had antibodies specific for both the known epitope as well as a peptide complementary to the epitope [84]. The group then injected mice with both La/SSB peptides

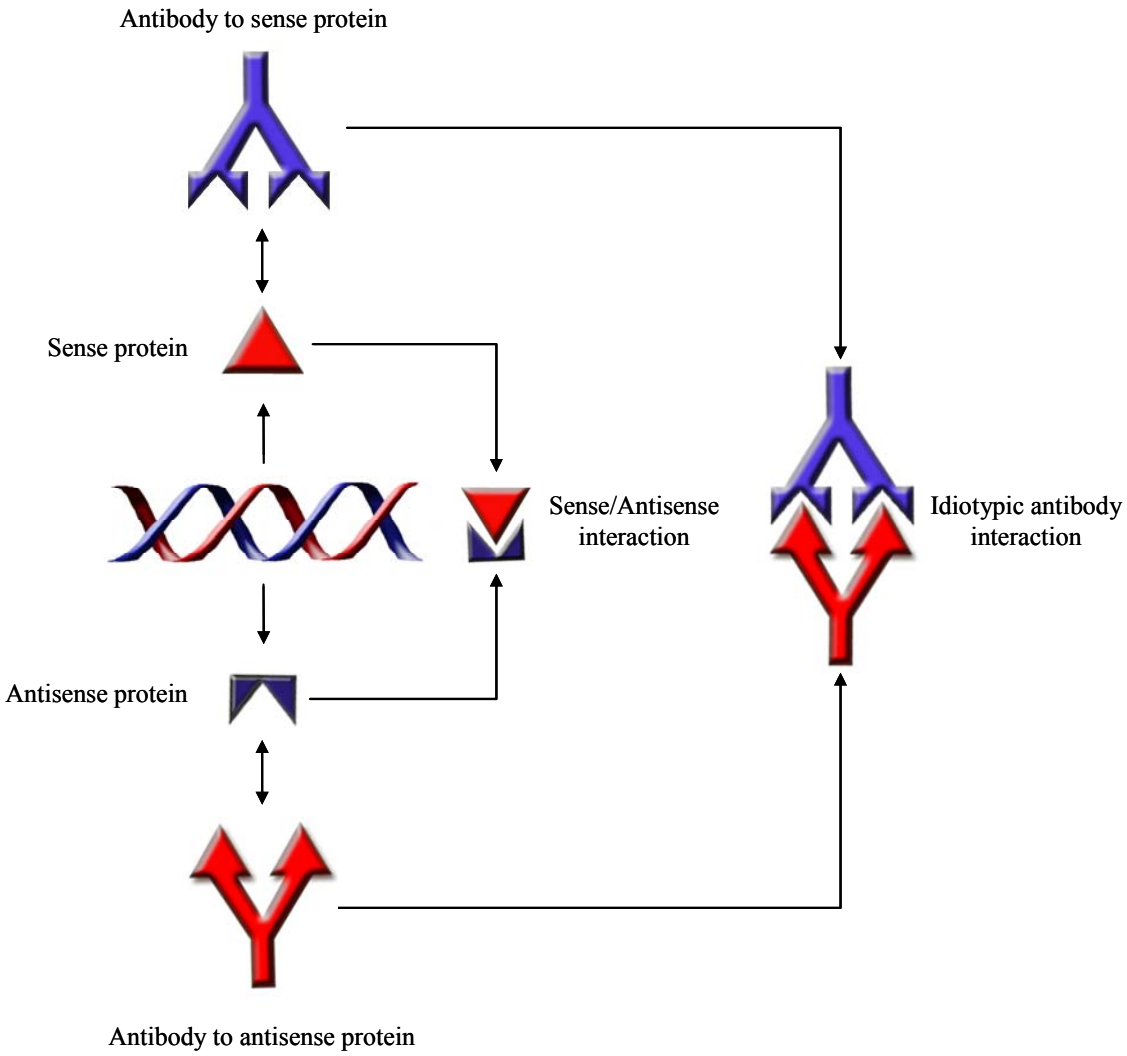

Figure 1.2. Complementary Proteins and the Idiotypic Network.
Complementary proteins, expressed in-frame off sense/antisense nucleic acid strands, specifically interact. Smith et al. showed that antibodies raised against a pair of complementary peptides (that interact) bind in an idiotypic fashion through their variable regions. Pendergraft et al. expanded upon this work by showing injection of an antisense peptide resulted in production of antibodies to both antisense peptide and sense peptide, thus demonstrating an intact idiotypic network and its relation to complementary proteins.

(both sense and complementary) and showed that antibodies to La/SSB were produced in both sense and antisense-immunized animals [85]. They also devised a novel ELISA to unmask idiotypic antibody relationships that were interfering with their test. In mice immunized with the complementary peptide they were initially unable to show a response to La/SSB protein. They hypothesized that anti-complementary peptide antibodies were binding to anti-La/SSB antibodies. To circumvent this problem, they heated sera samples at 55°C to disrupt antibody/antibody interactions and then added complementary peptide to block binding between the two antibodies. The samples were re-tested and they showed that the mice immunized with complementary antibody did in fact have antibodies to La/SSB. In addition, they were able to show that T-cell help was required for anti-idiotypic antibody development [86].

AUTOANTIGEN COMPLEMENTARITY

The theory of autoantigen complementarity was conceived based partly on the fact that a subset of PR3-ANCA patients were shown to have antibodies that reacted with a recombinant, complementary-PR3 protein [$cPR3^{(105\text{-}201)}$] [49]. In agreement with the results seen by Smith et al. [66], antibodies to PR3 were separate and distinct from antibodies to $cPR3^{(105\text{-}201)}$ and the two sets of antibodies bound in an idiotypic fashion. In addition, mice injected with $cPR3^{(105\text{-}201)}$ developed not only antibodies to cPR3 but also antibodies to human PR3.

The theory of autoantigen complementarity asserts that it is not the autoantigen, but a peptide or protein *complementary* to the autoantigen that drives autoimmune disease. Specifically, an antibody response against a protein complementary to an autoantigen triggers

an anti-idiotypic antibody response (in agreement with Jerne's network theory). This anti-idiotypic antibody then reacts with the autoantigen resulting in autoimmune disease (Figure 1.3).

Where do complementary proteins come from? The theory proposes that the complementary proteins could either be produced endogenously (by aberrant transcription and translation of antisense message) or introduced exogenously by a microbial pathogen. In fact, there is evidence to support both of these routes. There are a large number of antisense transcripts identified in the human genome [87], and in fact a protein encoded by the antisense strand of a ubiquitously expressed gene has been identified in kidney cancer [88]. Antisense transcripts from the PR3 gene were found in 10 of 22 PR3-ANCA patients tested, while no antisense PR3 transcripts were found in a group of SLE patients or healthy controls [49]. In addition to being produced by the host, complementary proteins (or their mimics) could be introduced by microbes and/or viruses. There are a number of bacterial and viral proteins that share sequence homology with proteins that are antisense to known autoantigens [89], including proteins from two microbes, *Staphylococcus aureus* and *Entamoeba histolytica*, which have homologies to $cPR3^{(105\text{-}201)}$ and have been most closely linked to the development of PR3-ANCA [90, 91].

ANTIGENIC COMPLEMENTARITY AND THE DEFINITION OF COMPLEMENTARY PROTEINS

Root-Bernstein et al. proposed the theory of antigenic complementarity to explain the induction of autoimmunity [92]. The theory posits that a pair of molecularly complementary antigens (where at least one of the antigens mimics a "self" protein) gives rise to a pair of

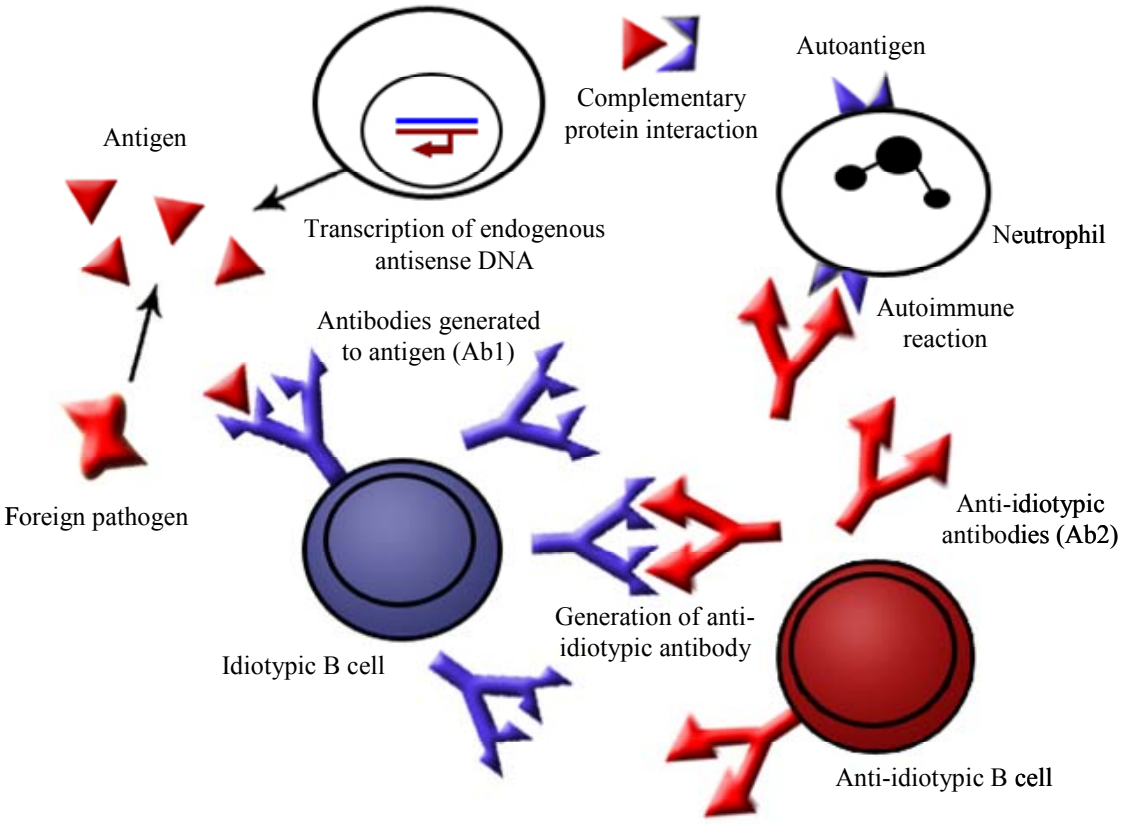

Figure 1.3. The Theory of Autoantigen Complementarity.
The theory posits that it is not the autoantigen, but a protein or peptide complementary to the autoantigen that drives autoimmune disease. The antigen (shown in red) is introduced through either transcription/translation of endogenous antisense DNA or by a foreign pathogen. This initiates the production of antibodies (Ab1, shown in blue). Through the idiotypic network, Ab1 then causes the production of anti-idiotypic antibodies (Ab2, shown in red) that in turn react with the autoantigen, in this case a protein expressed on the surface of neutrophils as in the case with ANCA disease. This theory is predicated on the fact that the antigen and the autoantigen have complementary shapes that result in binding.

molecularly complementary immune responses that attack each other along with a tissue or organ in the body (Figure 1.4). This theory is similar to the theory of autoantigen complementarity in that it proposes complementary proteins are involved in induction of autoimmune disease; however there is an important difference in how a complementary protein pair is defined. The theory of autoantigen complementarity uses the Mekler definition of complementarity, that is, a peptide or protein produced, in-frame, from the antisense strand of the coding gene. Root-Bernstein et al. define complementary proteins as those able to bind to each other (molecularly complementary surfaces) and capable of inducing the production of complementary antibodies (or T cells) that act like idiotype-anti-idiotype pairs without the necessity of being encoded by sense-antisense codons [92]. Both definitions of complementarity are valid, with the Mekler definition being more stringent while the Root-Bernstein definition allows for a broader group of protein-protein pairs to be considered as complementary. The theory of antigenic complementarity also proposes that the sets of complementary proteins are likely to be microbial and/or viral in nature, and requires that only if one of these proteins is sufficient enough to "self" does an autoimmune reaction occur.

Some evidence exists to support antigenic complementarity. Root-Bernstein et al. used the autoimmune disease idiopathic thrombocytopenia purpura (ITP) as a case study for their theory [92]. ITP is characterized by autoantibodies to platelet glycoprotein 1b (pgp 1b) and von Willebrand's factor (VWF) [93]. pgp 1b and VWF bind to each other during the normal blood coagulation cascade and their binding regions have been well characterized (i.e., they are molecularly complementary) [94]. Root Bernstein et al. showed that antibodies to VWF bind to antibodies to pgp 1b (i.e., the molecular complementarity between the two

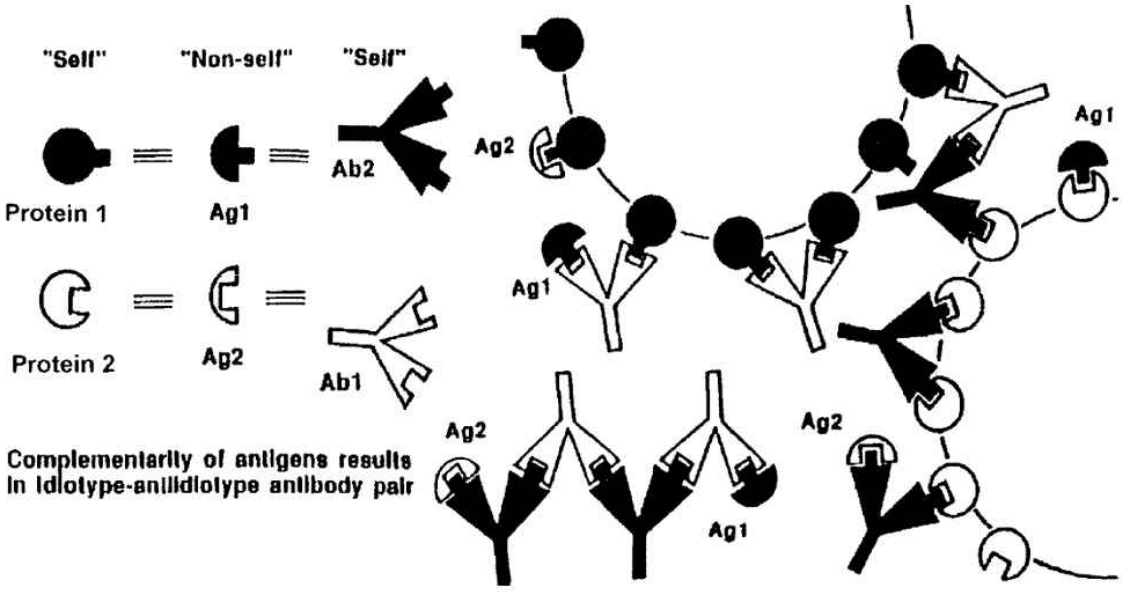

Figure 1.4. The Theory of Antigenic Complementarity.
The theory posits that it is a pair of molecularly complementary antigens that induce molecularly complementary antibody (or T-cell) responses, i.e. having an idiotypic relationship, but with both antibodies being idiotypic. These complementary antibodies will bind to each other and their respective antigens to create circulating immune complexes. Each antibody will also treat the other as "nonself", resulting in breakdown of the self-nonself distinction and an immunological civil war will be initiated. If one or both of the antigens are mimics of a self determinant then this civil war will spread to attack host tissue. Adapted from *Root-Bernstein et al*, *Clin Dev Immunol 2006*

proteins is reflected in the antibodies) [92]. They also showed that antibodies to cytomegalovirus (CMV) bound to antibodies to group A streptococcus (GAS), two infectious agents that have been associated with the onset of ITP [95, 96]. Proteins from CMV and GAS show significant homologies to both regions of VWF and pgp 1b that are involved in the binding of VWF and pgp 1b. Lastly, antibodies to GAS bind antibodies to VWF, and antibodies to CMV bind antibodies to pgp 1b. Taken together, these data fit their model; however showing this system functions in an animal model of disease is lacking.

PROJECT GOALS AND OUTCOMES

Knowing that patients have circulating antibodies to a protein complementary to PR3, we hypothesized that proteins complementary to PR3 could be purified and identified *in vivo*. Specifically, the objectives of the research presented here were to identify proteins that are complementary to PR3, to determine if these protein(s) had any functional or clinical significance, and whether these proteins could be implicated in the etiology of PR3-ANCA vasculitis. The hypothesis was that proteins complementary to PR3 could be identified in either patient's sera or tissue samples, these proteins would interact with PR3, and multiple patients would have antibodies specific to these complementary proteins.

Chapter II of this dissertation discusses the discovery of complementary-PR3 proteins in PR3-ANCA patients. One of the complementary proteins identified was plasminogen, a 90 kDa protein that circulates as the immature form of plasmin, the serine protease responsible for fibrin clot dissolution. Along with plasminogen, we discovered a microbial protein from Psuedomonas, Protein F, which reacted with our anti-complementary PR3 antibody and was found in two separate PR3-ANCA patients.

Chapter III of the dissertation will discuss the identification of a novel autoantibody to plasminogen in a subset of PR3-ANCA patients. These autoantibodies were not seen in a group of healthy control subjects, MPO-ANCA patients, or patients who had idiopathic thrombotic events. The antibodies altered normal plasminogen activity in two separate *in vitro* assays. In addition, these antibodies were found most often in patients who had suffered a thrombotic event.

The dissertation will conclude with the epilogue, where the research will be discussed with what is currently known and future directions for additional research will be offered.

CHAPTER II

T cell Responsiveness to Complementary-PR3 Protein: A Pathogenic Role for Autoantigen Complementarity in ANCA Disease

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ABSTRACT

We discovered that patients with PR3-ANCA vasculitis have antibodies reactive against a protein complementary to the autoantigen PR3 [$cPR3^{(105\text{-}201)}$]. Investigations into the etiology and consequences of these anti-$cPR3^{(105\text{-}201)}$ antibodies led to the proposal that complementary proteins are involved in inciting autoantibody production, as described in the theory of autoantigen complementarity. The present studies indicate that $CD4^+$ T_H1 cells from PR3-ANCA vasculitis patients (n = 26) versus healthy controls (n = 34) exhibit increased proliferation ($P = 0.0014$) and IFN-γ expression when stimulated with $cPR3^{138\text{-}169}$ peptide ($P = 0.0002$), but not a scrambled peptide ($P = 0.6$ and $P = 0.3$, respectively). This response was not observed in T cells from MPO-ANCA patients. Reactivity to smaller, overlapping fragments of $cPR3^{(138\text{-}169)}$ ruled out the possibility that $cPR3^{(138\text{-}169)}$ peptide functions as a superantigen. Ranked linear regression analysis indicated a likelihood that anti-$cPR3^{(105\text{-}201)}$ antibodies and $cPR3^{138\text{-}169}$-specific T cells coexist within an individual ($P = 0.009$), consistent with an immunological history of an encounter with a complementary-PR3 protein. Consideration of potential contributions of complementary protein pairs in autoimmune diseases could revolutionize the approach for exploring pathogenic mechanisms.

INTRODUCTION

The concept of complementary protein pairs was first proposed by L.B. Mekler in the late 1960s [97, 98]. He proposed that information embedded within the genetic code could identify proteins that would pair in nature: proteins from sense codons bind proteins from their antisense codons. Skeptics of the validity of this idea are gradually realizing that experimentally this works and many researchers have discovered protein partners by utilizing complementary sequences coded by antisense codons (reviewed in [57, 99]). Of course, not all proteins that form complexes meet Mekler's definition of a complementary pair. Researchers debate what characteristics truly constitute a complementary pair. A recent and broader definition of a complementary pair states that two proteins are complementary if they are capable of stereospecific binding and inducing an idiotype-antiidiotype antibody response, thus eliminating the restriction of sense and antisense codons [92]. The mechanistic basis for the natural affinity for complementary protein pairs remains largely speculative [64, 92], however, it is thought that inverted hydropathy may be a driving force [52].

We became involved in studying complementary proteins after an unanticipated observation that patients with PR3-specific antineutrophil cytoplasmic autoantibodies (PR3-ANCA) had antibodies against a complementary-PR3 protein. Follow-up on these initial observations required production of a recombinant complementary-PR3 protein, designed by inserting nucleotides 315 to 618 (size predetermined by STOP codons) of PR3 cDNA into a vector in a flipped orientation. The recombinant complementary-PR3 protein, termed $cPR3^{(105\text{-}201)}$, represents the middle third of the sense-PR3 protein (aa 105-201). $cPR3^{(105\text{-}201)}$ protein formed a complex with native PR3 blocking its proteolytic activity [49]. Using this

reagent, we proved that anti-*cPR3*$^{(105\text{-}201)}$ antibodies were unique and that they bound PR3-ANCA to form an idiotypic pair.

We put forward the theory of autoantigen complementarity, which proposes that inciting antigens for autoimmune diseases are not the autoantigens, but instead proteins complementary to the relevant autoantigens [49]. The first event is an antibody response that targets a complementary protein; subsequently this antibody triggers an anti-idiotypic response. It is the anti-idiotypic antibody that targets the autoantigen.

These earlier studies focused on complementary proteins and their antibody-producing B cells. To date, no one has purposefully searched in humans for T cells that respond to complementary protein counterparts of known autoantigens. What has been done in PR3-ANCA disease is a search for PR3-specific reactive T cells, but with little satisfaction. The problem has been the lack of statistical differences between patients and healthy controls [100-106]. These data raise the question of why on the one hand we can identify peptide-specific, disease associated-IgG antibodies [107], but on the other hand have difficulty identifing the corresponding peptide-specific T cells [108]. A new approach is needed, and investigations into the potential contributions of complementary proteins provides a novel alternative. In support, a report published in the year 2000 proposed that difficulties identifing disease-associated PR3-specific T cells could be explained if a protein complexed to PR3, and not PR3 itself, was what actually induced T cell help for PR3-specific B cells [104]. One suggestion is that PR3 may be complexed with a "complementary protein". Complementary antigens can form a molecular complex [52, 109] and this complex could have a unique structure that appears foreign. Thus, processing of such a complex might result in a range of primary antibodies and/or T cell reactive clones:

some against the antigenic complex and some biased towards the individual components. Albeit, immunological responses incited by a complementary protein not complexed to its sense counterpart must be equally considered at this point.

If patients with PR3-ANCA have experienced an immunological encounter with a *cPR3*-like antigen, then the T cells involved in that encounter may still be present and identifiable. Herein we establish the first human correlate between T cell responses and complementary proteins.

MATERIALS AND METHODS

Patients

All subjects gave written informed consent and participated in the study according to the guidelines of the UNC Institutional Review Board (IRB # 97-MED-44). The study included 9 females/17 males; mean age 49.8 yrs (26-79 yrs); 3 blacks, 22 Caucasians, 1 Asian (Table 1). Mean of PR3-ANCA titers across samples was 51.7 (range: 3.2-170.0). Healthy controls were recruited on site for blood donations (n = 34). The disease control group of seven MPO-ANCA patients included 5 females /2 males, mean age 45 yrs (21-65 yrs), 1 black, 5 Caucasians and 1 Asian. Limits on the amount of blood obtainable per donation required that the different methodologies in this study be done in tandem. Twelve of 26 patients donated blood more than once during the study's two-year period. Studies for anti-$cPR3^{(105\text{-}201)}$ antibody reactivity required the use of banked sera samples. Healthy control sera were from approved kidney transplant donors (n = 12).

Proteins and synthetic peptides

Recombinant $cPR3^{(105\text{-}201)}$ protein was produced as previously described [49]. The sequence of the protein is:

DAGLAARDESANVMWPAEEGDHGDIELLQDLGWGVVGTHAAPAHGQALGAVGH

WLVLLWQLDCGDGGTEVGWAAQLDEENVVQFVLRVVVVQKHLSHREVLLGGLLR

PHVVGSEHHVHQALGYVPQAVRGRQHEAG ($cPR3^{(138\text{-}169)}$ peptide underlined).

Synthetic peptides from Alpha Diagnostic (San Antonio, TX) included:

$cPR3^{(138\text{-}169)}$: N-DLGWGVVGTHAAPAHGQALGAVGHWLVLLWQL-C (32aa)

Fragment 1 - *cPR3*$^{(138\text{-}153)}$: N-DLGWGVVGTHAAPAHG-C (16aa)

Fragment 2 - *cPR3*$^{(146\text{-}161)}$: N-THAAPAHGQALGAVGH-C (16aa)

Fragment 3 - *cPR3*$^{(154\text{-}169)}$: N-QALGAVGHWLVLLWQL-C (16aa)

Sense-PR3$^{(138\text{-}169)}$: N-QLPQQDQPVPHGTQCLAMGWGRVGAHDPPAQV-C (32aa)

Scrambled peptide: N-LWAGDWVALGLGAWLAGLHVHAQTPHVQVGGL-C (32aa)

Purchased PR3 (Wieslab AB, Lund, Sweden) was passed over an Extracti-Gel AffinityPak detergent-removing column (Pierce, Rockford, IL) and heat inactivated (HI) (100^{o}C/10 min) [106] to linearize the protein [103]. Recall antigen mixture contained tetanus toxoid (2 μg/ml) and diphtheria toxin (2 μg/ml) (LIST LABS, Campbell, CA), plus 15 μg/ml of candida (Allermed Lab, San Diego, CA). Other agents: concavalin A (Con A) (1 μg/ml), phorbol 12-myristate 13-acetate (PMA) (25 ng/ml), and ionomycin (1 μg/ml) (Sigma, St. Louis, MO).

Cell stimulations

Blood was collected into sodium heparin CPT^{TM} Cell Preparation tubes (BD Vacutainer®, Franklin Lakes, NJ) and peripheral blood mononuclear cells isolated per instructions. Stimulants included peptides (2-25 μg/ml), HI-PR3 (2-10 μg/ml), recall antigen mixture and either ConA or PMA plus ionomycin. Peptide solvent dimethyl sulfoxide (DMSO) was added to controls.

CFSE Assay

Cytoplasmic proteins were fluorescently labeled with carboxy-fluorescein diacetate, succinimidyl ester (CFSE) (0.1 μM) for 15min (Molecular Probes, Eugene, OR, USA).

Subsequent proliferation in the absence of CFSE results in decreased fluorescence intensity by ½ with each cell division. Cells were cultured at $1x10^6$ cell/ml 6 days with proteins (10 µg/ml) or peptides (25 µg/ml). $CD3^+$ cells were labeled with phycoerythrin (PE)-mouse anti-human CD3 monoclonal antibody (BD PharMingen, San Diego, CA) and analyzed by FACScan linked to a CELLQuest software system (Becton Dickinson Immunocytometry Systems, San Jose, CA). The cell division index (CDI) is based on 5000 $CFSE^{bright}$ $CD3^+$ cells as previously described [110].

$$CDI = \frac{\left(\frac{CFSE^{dim}}{CFSE^{bright}/5000}\right)^{treated}}{\left(\frac{CFSE^{dim}}{CFSE^{bright}/5000}\right)^{untreated}}$$

Enzyme-linked immunospot assay (ELISPOT)

Cells were plated at 2 x 10^6 cells/ml in 100µl in triplicate on MultiScreen 96-Well Filtration Plates (Millipore, Bedford, MA) and coated with anti-human IFN-γ monoclonal antibody (Pierce). Treatments included peptides (5 µg/ml), HI-PR3 (2 µg/ml), recall antigens and Con A. IFN-γ releasing cells were detected with biotinylated-mouse anti-human IFN-γ antibody (Pierce) (2µg/ml), streptavidin (SouthernBiotech, Birmingham, AL) and AEC solution, containing a 3-amino-9-ethylcarbazole tablet, N,N-Dimethyformamide and hydrogen peroxide (Sigma). Data were analyzed using ImmunoSpot reader, ImmunoSpot 3 software, version 3.2 (Cellular Technology Ltd., Cleveland, OH).

Intracellular cytokine production of $CD4^+$ and $CD8^+$ cells

Cells ($0.8x10^6$/ml/well) were cultured with HI-PR3 (10 μg/ml) or peptides (5 μg/ml) for four days. PMA and ionomycin (6 hrs) served as a positive control. Brefeldin A (Sigma) was added (10 μg/ml) for 4hrs. Cells were fixed using FACS Lysing Solution and FACS Permeabilizing Solution 2 (Becton Dickinson) and were labeled with FastImmune anti-human IFN-γ FITC antibody (BD PharMingen) and anti-human CD4- or CD8-PerCP labeled antibodies (BD Immunocytometry Systems) and analyzed by FACScan.

Detection of anti-$cPR3^{(105\text{-}201)}$ *antibodies in sera by ELISA*

High-binding plates (Coster, Cambridge, MA) were coated overnight at 4^oC with recombinant $cPR3^{(105\text{-}201)}$ protein (5 μg/ml). Sera was added (1:100) and reactive antibodies were detected with alkaline phosphatase goat anti-human antibody (Chemicon, Temecula, CA) plus alkaline phosphatase Substrate (Bio-Rad, Hercules, CA, USA) and read on a VERSAmax microplate reader (Molecular Devices, UK). Values are percent of positive control (rabbit anti-his-tag antibody) (Santa Cruz Biotech, Santa Cruz, CA, USA) compared to the mean plus two standard deviations of healthy controls.

Statistical analysis

The Wilcoxon rank sum test was used as a nonparametric alternative to the two-sample *t*-test for analysis of T cell responses to compare patients and healthy controls. Ranked linear regression analysis was used to determine associations between complementary protein responsive T cells and reactive antibodies.

RESULTS

Patient study group

Patients with biopsy-proven PR3-ANCA vasculitis (n = 26) were identified through the Glomerular Disease Collaborative Network between January 2004 and June 2006 (Table 2.1). To avoid the potential of T cell anergy due to immunosuppressive therapies, the patient enrollment was limited to those in remission, slightly active disease on maintenance drugs, and newly diagnosed patients before aggressive treatment. Based on the Birmingham Vasculitis Activity Score (BVAS) 2003, 25 samples were from patients in remission (BVAS = 0) and 17 samples were from patients with active disease (BVAS > 0) (Table 2.1). Because of limitations on the amount of blood obtainable per patient-donation, inclusion in the different analyses required that patients donate more than once during the study's two-year period.

Proliferative response of cPR3$^{(138\text{-}169)}$ *peptide reactive T cells*

If patients with PR3-ANCA vasculitis have experienced an immunological encounter with a complementary-PR3-like protein, they should possess T cell pools of previously activated, differentiated cells that now exist as long-lived memory cells. The particular amino acid sequence of *cPR3*$^{(138\text{-}169)}$ peptide used in this study was first identified as an epitope of patients' antibodies during a screen of a bacterial expression library [49]. This specificity was confirmed by mass spectrometry (data not shown). $CD3^+$ T cell subsets from patients exhibited increased proliferation upon encounter with *cPR3*$^{(138\text{-}169)}$ peptide compared

Table 2.1. Clinical and Demographic Characteristics of Patients

Patients (n = 26)	*Samples (n = 41)*	*Sex*	*Race*	*Age (years)*	*Clinical Diagnosis*	*BVAS Score*	*Treatment*
A	1	M	W	31	WG	0	MMF
	2					0	MMF
	3					0	MMF
B	1	M	B	50	WG	0	AZA, GC
	2					0	AZA, GC
C		M	W	58	CSS	0	AZA
D		M	W	42	MPA	0	MMF
E		M	W	72	WG	0	CYC, GC
F		F	W	47	MPA	0	MMF
G	1	M	W	69	WG	0	CYC, GC
	2					0	AZA, GC
H	1 (2 tests)	F	W	66	WG	0	GC
I	1	M	W	26	MPA	0	AZA, GC
	2					0	AZA, GC
J	1	M	W	38	MPA	0	Off therapy
	2					0	Off therapy
K	1	M	W	57	MPA	0	AZA
	2					0	AZA
L		M	W	64	MPA	0	CYC
M	1 (2 tests)	M	B	32	WG	0	Off therapy
N		M	W	26	WG	0	Off therapy
O	1	F	B	55	WG	3	AZA
	2					3	AZA
	3					0	AZA
	4					3	AZA
P	1	M	W	34	WG	5	GC
	2					1	MMF, GC
Q	1	F	W	79	MPA	2	MMF
	2					0	MMF
R	1	M	W	59	WG	3	CYC
	2					0	Off therapy
S	1 (2 tests)	F	W	56	WG	7	AZA, GC
T		F	W	56	WG	6	MMF*
U		M	Other	31	WG	5	MMF
V		M	W	47	WG	3	MMF, GC
W		F	W	26	WG	3	AZA, CsA
X		M	W	50	MPA	3	MMF, CsA, GC
Y	1	F	W	63	WG	12¶	CYC, GC
	2					3	AZA, GC
Z	1	F	W	59	MPA	11¶	CYC, GC
	2					0	MMF, CsA

MMF: Mycophenolate Mofetil; CYC: Cyclophosphamide; AZA: Azathioprine
GC: Glucocorticoids; CsA: Cyclosporine; WG: Wegener's Granulomatosis
MPA: Microscopic Polyangiitis; CSS: Churg Strauss Syndrome;
¶Onset of disease
*Status post Rituximab therapy

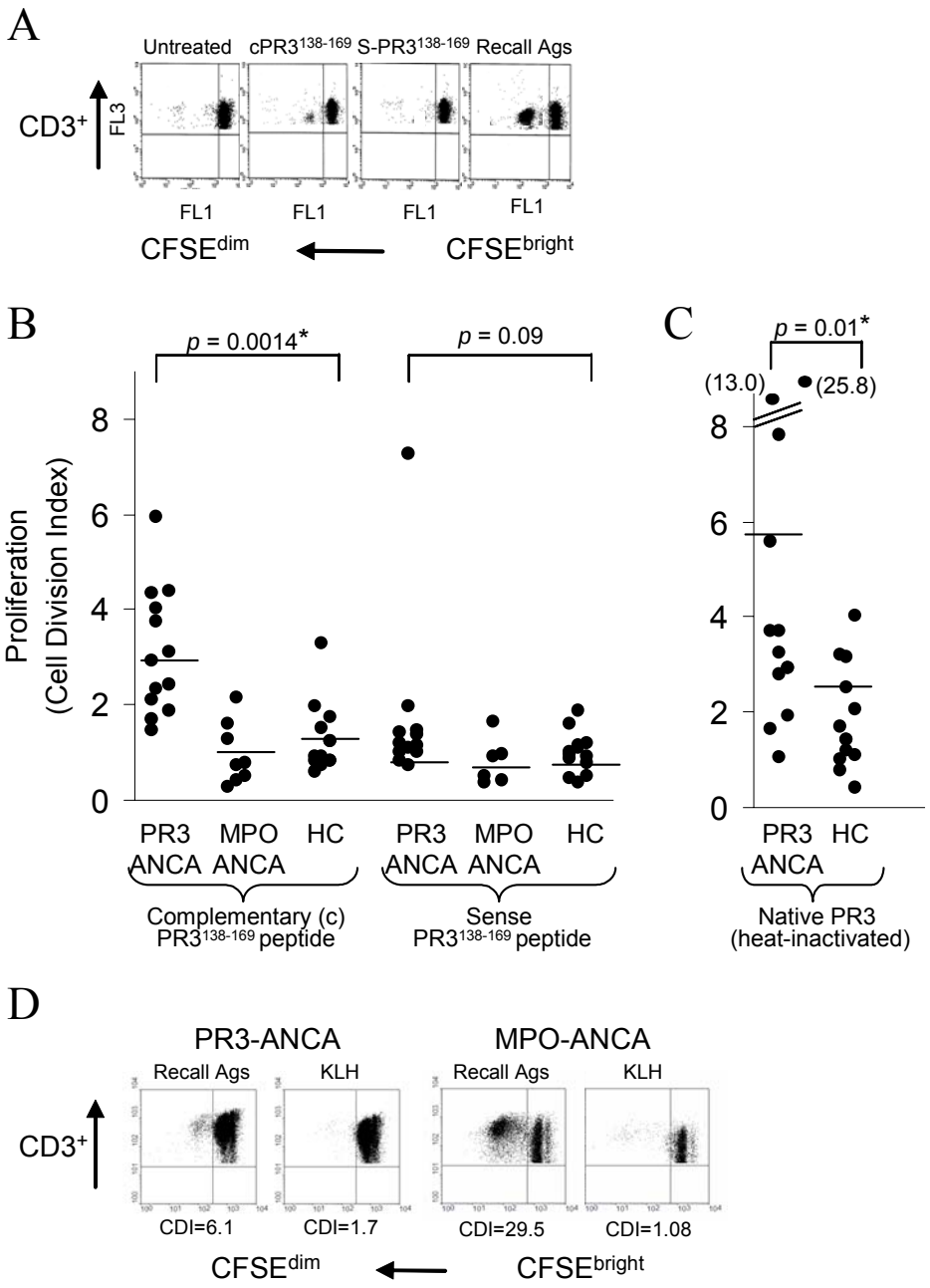

Figure 2.1. PR3-ANCA Patients T cells Respond to *cPR3*$^{(138-169)}$
(A). A representative example of flow cytometric data; $CD3^+$ T cells were gated and analyzed for the presence of a $CFSE^{dim}$ subset (upper left quadrant). (B & C) Proliferative response of $CD3^+$ T cells of vasculitis patients with PR3-ANCA, MPO ANCA and healthy controls (HC) after antigen stimulation. Combined data were plotted as cell division index (CDI). Solid horizontal lines indicate mean values. Comparisons between groups were done using the Wilcoxon Ranked Sum Test. (D) Positive T cell response to recall antigen coincident with non-responsiveness to KLH indicates responding cells are memory cells versus naïve cells.

to healthy controls (n = 13) (Wilcoxon rank sum test, P = 0.0014) (Figure 2.1B). A proliferative response to heat-inactivated-PR3 was detected in patients (P = 0.01) (Figure 2.1C), although no differences were found upon encounter with sense-PR3$^{(138\text{-}169)}$ peptide (P = 0.09). The CDI of responses to recall antigens was also similar comparing 9.4 ± 5.3 versus controls 11.0 ± 11.5 (P = 0.79). The percent of background proliferation was similar comparing patients (3.0 ± 4.2) versus controls (2.0 ± 2.0).

The proliferative response to *cPR3*$^{(138\text{-}169)}$ peptide occurred without additional costimulation with cytokines, suggesting that these T cells had a memory cell phenotype [111]. Indeed, no significant proliferation in response to KLH was detected in two PR3-ANCA patients and seven MPO-ANCA patients (mean CDI: recall antigens = 6.55 ± 8.9; KLH = 1.10 ± 0.49) (Figure 2.1D), indicating that under the culture conditions used only previously primed but not naïve T cells are detected.

T cells produce IFN-γ in response to cPR3$^{(138\text{-}169)}$ *peptide*

cPR3$^{(138\text{-}169)}$ stimulated an IFN-γ response in patients' T cells but not in cells from healthy controls (Wilcoxon rank sum test, P = 0.0002) (Figure 2.2A). Patient responses to sense-PR3$^{(138\text{-}169)}$ peptide was comparable to healthy controls (P = 0.12) as was the responses to PR3 (P = 0.19) and scrambled *cPR3*$^{(138\text{-}169)}$ peptide (P = 0.35) (Figure 2.2A). There was a tendency for patients' samples to be less responsive to the recall antigen controls, a reflection of their generally poor state of health and use of a variety of medications; however, statistically there was no difference between patients and healthy controls (Figure 2.2B). Dose-dependent responses of three of these patients versus healthy controls confirmed the

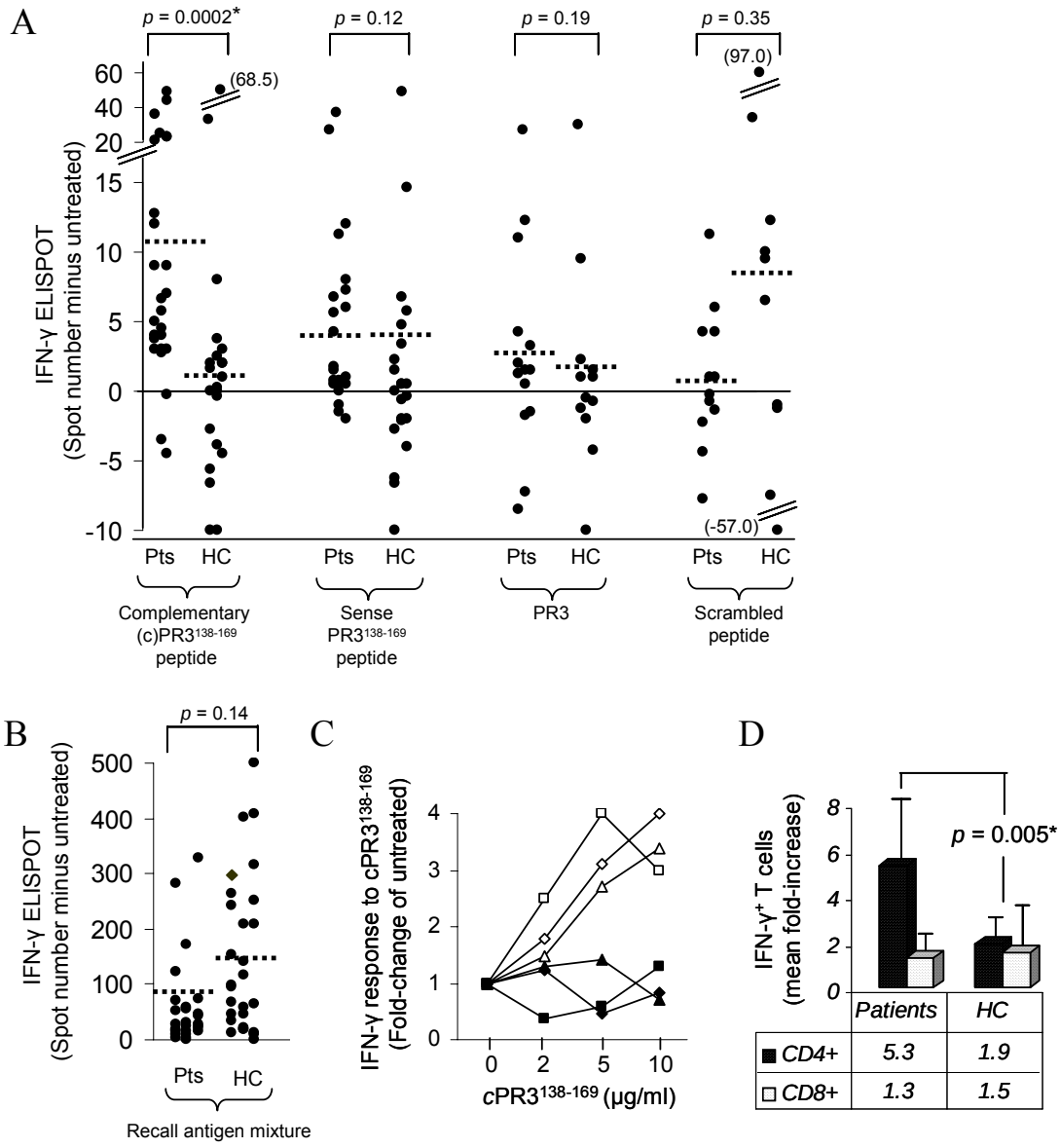

Figure 2.2. PR3-ANCA Patient T cells Produce IFN-γ in Response to *cPR3*$^{(138\text{-}169)}$
(A) T cell IFN-γ production in response to antigens. ANCA vasculitis patients (Pts) and healthy controls (HC) were analyzed for cytokine production by ELISPOT. Results show increased responses in Pts against the *cPR3*$^{(138\text{-}169)}$ peptide (data indicate spot number with antigen minus spot number without antigen). (B) Responses to recall antigens (mixture containing tetanus toxoid, diphtheria toxin, and candida). (C) Dose- dependent specificity of response to *cPR3*$^{(138\text{-}169)}$ peptide. Results are shown as mean spot number with antigen divided by mean spot number without antigen. Three patients (open symbols) and three healthy controls (closed symbols) were analyzed for IFN-γ release by ELISPOT in varying concentrations of peptide. (D) $CD4^+$ T cells were the IFN-γ responsive cells. Data represent the mean increase of treated cells compared to untreated of patients and healthy controls. Comparisons between groups were done using the Wilcoxon Ranked Sum Test.

specificity of an IFN-γ-response to this peptide (Figure 2.2C). The *cPR3*$^{(138\text{-}169)}$ peptide IFN-γ-responders were of the $CD4^+$ subset with a mean increase in patients of 5.3 ± 3.5-fold compared to healthy controls at 1.9 ± 0.75 (Wilcoxon ranked sum test, $P = 0.005$) (Figure 2.2D). IFN-γ-positive $CD8^+$ cells were not increased (mean of 1.3 ± 1.02) compared to healthy controls (mean of 1.5 ± 0.67) ($P = 1.0$) (Figure 2.2D).

Specificity of responses to fragments of cPR3$^{(138\text{-}169)}$ *peptide*

cPR3$^{(138\text{-}169)}$ peptide bears some homologies to a number of bacterial proteins [49], and it was questioned whether this 32aa peptide had characteristics similar to a pathogen-derived superagonist. Three 16aa overlapping peptide-fragments were tested for stimulatory characteristics with the supposition that superantigen-like sequences would bias reactivity toward one fragment. IFN-γ responses were random i.e., no *cPR3*$^{(138\text{-}169)}$ specific sequences common to all of patients (Table 2.2). Of healthy controls (n = 12), nine individuals' T cells were non-reactive, while two had greater than five spots on the assay against fragment 1 and one reacted with fragment 2. The data indicate that *cPR3*$^{(138\text{-}169)}$ is not a superantigen.

Individual variability among longitudinal samples

A graphical representation of each patient's T cell responsiveness over time provides a look at the potential for variable outcomes (Figure 2.3A). Patient A was positive in all three assays with the proliferation studies (2004), FACS (2005), and ELISPOT (2006). However, patient K was negative in 2004 for proliferation, and positive in 2005 for ELISPOT assay. Explanations for variability are not obvious as both patients were in remission and drug regimens remained consistent. Statistical comparisons of individuals in

Table 2.2. Responses to *cPR3*$^{(138-169)}$ Peptide Fragments

Patients	****Spot number cPR3$^{138-153}$ (Fragment 1)***	****Spot number cPR3$^{146-161}$ (Fragment 2)***	****Spot number cPR3$^{154-169}$ (Fragment 3)***
B	72.6	6.6	24.2
T	29.0	17.0	12.0
V	18.0	≤untreated	14.0
R	9.0	5.4	5.4
A	6.8	2.5	2.5
U	5.5	3.5	≤untreated
P	2.5	≤untreated	≤untreated
O	32.2	41.5	41.5
J	5.5	10.5	2.1
D	6.8	9.8	17.3
Q	26.0	16.0	29.0
C	1.3	0. 5	5.8
Controls			
H-A	≤untreated	≤untreated	≤untreated
H-B	1.3	≤untreated	≤untreated
H-C	≤untreated	≤untreated	≤untreated
H-D	7.3	4.7	2.0
H-E	≤untreated	≤untreated	1.5
H-F	≤untreated	2.0	≤untreated
H-G	≤untreated	≤untreated	≤untreated
H-H	≤untreated	≤untreated	≤untreated
H-I	4.5	25.5	≤untreated
H-J	12.5	0.5	2.5
H-K	≤untreated	≤untreated	≤untreated
H-L	1.5	≤untreated	1.0

**The ELISPOT data are expressed as positive spots of treated wells minus spots on untreated wells.

cPR3$^{(138-169)}$ DLGWGVVGTHAAPAHGQALGAVGHWLVLLWQL
Fragment 1 DLGWGVVGTHAAPAHG
Fragment 2 THAAPAHGQALGAVGH
Fragment 3 QALGAVGHWLVLLWQL

remission who showed a positive T cell response (BVAS = 0; 11 of 13) (patients A through N - Table 2.1) versus those who were not in remission (with BVAS score > 0; 10 of 13) (patients O through Z - Table 2.1) indicated an equal distribution within the two groups (Fisher's exact test $P = 0.99$). Moreover, statistical comparisons of samples taken at times of remission (n = 25), compared to active disease (n = 16) (Table 2.1; Figure 2.3B) gave similar results showing an equal distribution of responders and non-responders (Fisher's exact test, $P = 0.74$). The patients with high BVAS scores were newly diagnosed on medications for only a few days. These patients responded to recall antigens and to *cPR3*$^{(138\text{-}169)}$ peptide. Those patients on high dose – long duration medication failed to respond to recall antigen controls and thus were non-informative. Other potential explanations such as type of medication (Table 2.1) or environmental related factors were not identifiable. Variability is inevitable and appears to be a common occurrence in studies of human subjects [104]. The validity of our results is substantiated by use of multiple methodologies and by repetitive patient samplings.

Coexistence of cPR3$^{(138\text{-}169)}$*-specific T cells with* cPR3$^{(105\text{-}201)}$*-specific antibodies*

A critical question concerning the functional consequences of *cPR3*$^{(138\text{-}169)}$ peptide-reactive $CD4^+$ T_H1 cells is whether they were responsible for *cPR3*$^{(105\text{-}201)}$-specific B cell maturation and antibody expression. Specifically, of the patients studied for T cell responses, is there any evidence that, at some point in time, they had circulating *cPR3*$^{(105\text{-}201)}$-specific antibodies? Stored sera samples were analyzed for the peak value for each individual (which in reality may not be the true physiological peaks due to limited samplings). Antibody reactivity was higher in patients calculated as percent of positive control (27.96 ± 21.20)

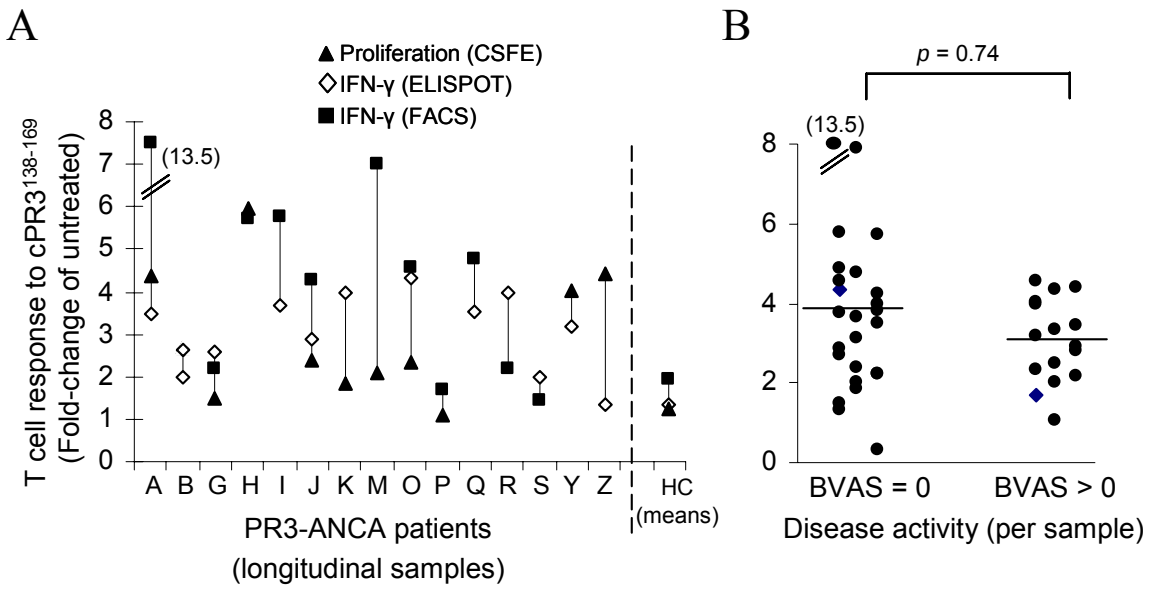

Figure 2.3. PR3-ANCA Patient Sample Variability and its Relationship to BVAS Score
(A) Sample variability. Detection of *cPR3*$^{(138\text{-}169)}$ peptide responsive T cells in patient samples donated over time during the course of the study for proliferative responses, FACS analysis of T cell subsets, and ELISPOT analysis for IFN-γ production. Mean value of healthy controls (HC) for each assay shown for comparison. (B) Evaluation of the effects of disease activity (based on BVAS score) on T cell responsiveness to *cPR3*$^{(138\text{-}169)}$ peptide.

compared to healthy individuals (15.59 ± 5.51) (Wilcoxon ranked sum analysis, $P = 0.05$). A ranked linear regression analysis indicated a likelihood of P value 0.0086 that, if patients had $cPR3^{(138\text{-}169)}$ peptide reactive T cells, they would also have the reactive antibodies. The data support the conclusion that a complementary-PR3 protein (or its mimic) was presented as a helper T cell epitope stimulating B cell maturation and antibody production.

DISCUSSION

The approach of exploring complementary protein pairs in autoimmune diseases offers a "breakthrough" in understanding mechanisms of pathogenesis. This is the first report of disease-related T cell responsiveness to a protein complementary to a known autoantigen. These *cPR3*$^{(138\text{-}169)}$-responsive cells were classified as $CD4^{+}$-T_H1 cells, which are capable of delivering signals for B cell maturation [112]. There was a significant correlation between the presence of anti-*cPR3*$^{(105\text{-}201)}$ antibodies and responsive T cells on an individual basis. These data are consistent with a complementary-protein-specific component in immunological events of PR3-ANCA vasculitis autoimmune disease.

A limitation when studying human T cells is that the only available sample is peripheral blood cells, unlike animial studies where spleens and lymph nodes are available. Using circulating cells, we found that the number of spots in the ELISPOT assay were less than published animal studies using spleens. Others report similarly low numbers of spots from human peripheral cells and propose this is expected for low-frequency reactive cells [113]. These are not unexpected as memory cells are thought to primarily reside in the spleen and peripheral lymphoid tissue, with low numbers of cells found in the circulation. Our efforts to expand the T cells in culture in order to increase the number of spots were unsuccessful. What is comforting is the degree of concordance between the proliferation studies and the ELISPOT assay with repetitive patient samplings. Even with these limitations, we successfully demonstrated a strong statistically significant response in patients compared to healthy controls.

A topic for discussion is why T cells specific for the PR3 autoantigen have been difficult to find at significant levels. Clayton and coworkers designed a system to determine

whether PR3-specific B cells require T cell help to produce antibodies using peripheral blood lymphocytes culture system. Their conclusions were that B cells from patients produce PR3-antibodies through a T cell independent pathway or through some non-specific B cell stimulation [114]. Nonetheless, PR3-specific T cells are identifiable. Consider for the moment that anti-idiotypic antibody processes are involved in the generation of PR3-specific antibodies. This possibility was supported when mice immunized with $cPR3^{(105\text{-}201)}$ peptide developed not only anti-$cPR3^{(105\text{-}201)}$ antibodies, but also antibodies that reacted with human-native PR3. These mouse anti-human-PR3 antibodies produced a cytoplasmic-staining pattern on human neutrophils identical to that produced by patients' PR3-ANCA. Thus, in these mice the derivation of the anti-human-PR3 reactive antibody must have occurred through an anti-idiotypic response incited by human-specific complementary protein [49]. The anti-idiotypic process is initiated with a T cell and B cell response against a PR3-complementary-protein. Antibodies can regulate each other by suppressing or augmenting the immune reaction in a manner that would perpetuate autoimmune disease [79, 115, 116]. An antibody is immunogenic by virtue of its non-germiline-encoded antigen-binding site. B cells are known to spontaneously display endogenous V region peptides on their HLA class II molecules and acivate $CD4^+$ T cells [117, 118]. Display of immunoglobulin-derived peptides (idiotopes) on APC HLA-II molecules can occur by several routes. Monocytes and dendritic cells phagocytize antigen-antibody complexes bound to surface F_c-receptors, and they directly phagocytize soluble antibodies through routine environmental sampling. Host antibodies are then degraded and loaded onto HLA II molecules and displayed on the APC surface in a manner similar to foreign antigens [119]. Alternatively, B cells endocytose antigens that ligate to surface immunoglobulin (the B cell receptor) and process these

proteins for display on HLA-II molecules [120-122]. How can this information be incorporated into understanding PR3-ANCA generation? Experimental evidence indicates that animals immunized with human complementary-PR3 protein not only develop antibodies reactive with the immunogen, but also development of human-specific PR3 antibodies. This has been observed in mice [49], rabbits, and chickens (unpublished data). Likewise, a research group who studies La/SSB-specific autoantibodies associated with Sjogren's syndrome and systemic lupus erythematosus found that mice immunized with the autoantigen's complementary-peptide-counterpart elicited antibodies against the immunogen and anti-idiotypic antibodies that reacted with the sense autoantigen [86]. It has been demonstrated in multiple autoimmunity animal models that anti-idiotypes, raised against autoantibodies, induced anti-anti-idiotypes that possessed characteristics of the initial autoantibodies and caused disease after immunization [123, 124].

A crucial question is the source of the actual complementary-PR3 proteins that triggered the immunological responses described here. Ongoing studies are addressing this by probing for proteins from patient material that react with our antibodies from rabbits immunized with complementary peptides. The possibilities remain that it could be carried in by a microbe with proteins homologous to the complementary protein [89, 125] or that patients aberrantly transcribe and translate it [49]. Somewhat encouraging, we have detected antisense transcripts in patients using an antisense specific primer for the reverse transcriptase reaction and PCR [49]. Whether these transcripts are, or even can be, translated is unclear, although there are reports of translated antisense transcripts [126].

A recent review prosposed that "complementary proteins, which occur naturally, or result from cellular dysfunction, might be more common than recognized currently. This

implies that the role of complementary proteins in autoimmunity merits increasing investigation"[127]. Understanding when and how complementary proteins initiate autoimmune disease will depend on discovering where these proteins come from. Nevertheless, there is enough evidence to warrant a closer look.

CHAPTER III

Isolation and Identification of Complementary-PR3 Proteins in PR3-ANCA Patient Plasmapheresis Fluid

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ABSTRACT

Biophysical interactions of proteins complementary to one another can provide a practical approach for discovery of novel autoimmune responses. Prior studies demonstrated that patients with anti-neutrophil cytoplasmic autoantibody small vessel vasculitis (ANCA SVV) mounted an immune response to proteins complementary to proteinase 3 (PR3). The current study demonstrates that a strategy capitalizing on principles of protein complementarity lead to the discovery of novel complementary-PR3 proteins. Plasma proteins from PR3-ANCA patients were analyzed for proteins complementary to PR3 by chromatography, SDS-PAGE, western blot analysis and mass spectrometry. Plasminogen and Protein F from pseudomonas were identified as putative complementary-PR3 proteins. Plasminogen is a substrate of PR3, indicative of interaction between these two proteins. In prior studies, immunization of mice with complementary-PR3 protein resulted in antibodies produced not only to complementary-PR3 but to human PR3 as well. These antibodies were shown to bind through their variable region; they were an idiotypic pair. A rabbit immunized with PR3 developed antibodies not only to PR3 but to plasminogen as well through the idiotypic network. Antibodies to PR3 were purified from chicken immunized with $cPR3^{(138-169)}$ peptide, demonstrating an intact idiotypic network that can function in either direction. Anti-$cPR3^{(138-169)}$ antibodies were shown to stain normal human leukocytes, cells that are known to bind large quantities of plasminogen. These studies demonstrate that the principles of protein complementarity can be utilized for the identification of previously unknown complementary proteins.

INTRODUCTION

Biochemical properties of complementary protein pairs and their respective antibodies are thought to contribute to autoimmunity. A serendipitous discovery by our research group, that patients with proteinase 3 (PR3)-specific anti-neutrophil cytoplasmic autoantibodies (PR3-ANCA) also had antibodies against a protein coded by the antisense strand of the PR3 cDNA [$cPR3^{(105\text{-}201)}$], led to the proposal that autoantigen complementarity is an underlying mechanism of this autoimmune disease [49]. The implications are that molecular complementarity approaches will lead to identification of other, and perhaps proximal, antigens in autoimmune disease. The goal of the present study was to isolate and identify a protein/s from patients' plasma that might have given rise to these anti-$cPR3^{105\text{-}201}$ antibodies.

The principles of complementary protein chemistry were first put forth in the 1960s proposing that a protein translated 5′ → 3′ from antisense RNA is a *complementary* counterpart of the protein coded by the sense RNA and that these two proteins have a natural affinity for binding [50, 98]. Since that time, investigators have repeatedly demonstrated this phenomenon exists [52, 99, 128]. A recent review cites numerous studies proving that sense proteins and their *complementary* counterparts have a natural affinity, and that increasing affinity correlates with increasing peptide length [57]. The concept of complementarity has been extended to explain antigen-antibody binding proposing that the variable region of an antibody is chemically complementary to its antigen [66, 81, 129]. Further extrapolations suggest that antibodies reactive with a sense protein and antibodies reactive with the respective complementary protein are complementary to each other and have an affinity for binding. This respective antibody pair is theorized to form an idiotypic pair [81]. In

conjunction, for over two decades, the idiotypic network has been implicated to be a component of autoimmunity by multiple researchers [79, 80, 116, 123, 124].

Of the autoimmune diseases we study which affect the kidney, one of the most profound is glomerulonephritis caused by ANCA specific for the neutrophil granule proteins PR3 or myeloperoxidase (MPO) [12, 33]. ANCA activate neutrophils and monocytes causing inappropriate release of granule constituents thus causing injury to vessel walls, in particular, the glomerular capillaries of the kidney and alveolar capillaries of the lung [21]. In efforts to gain insights into disease causation, we propose to identify the antigen(s) that gave rise to antibodies reactive with $cPR3^{(105\text{-}201)}$ recombinant protein found earlier in this patient group. Because plasmapheresis is often a treatment of choice, protein-rich material from acutely active patients was available for probing for putative *cPR3* proteins. Plasminogen and Protein F from Pseudomonas were shown to be reactive with chicken and rabbit anti-$cPR3^{(138\text{-}169)}$ antibodies. Anti-PR3 antibodies were purified from a chicken immunized with complementary PR3 peptide, demonstrating an intact immunological idiotypic network in yet another species. Plasminogen and PR3 were shown to interact; in fact plasminogen is a previously unrecognized substrate of PR3. In addition, a rabbit immunized with human PR3 developed antibodies not only to PR3 but to plasminogen as well, further evidence of a complementary relationship between the two proteins. Protein complementarity has been utilized to discover a novel protein-protein interaction and could provide a new means for identifying other such interactions.

MATERIALS AND METHODS

Antigens/Antibodies/Reagents

Complementary-PR3 peptide corresponding to PR3 residues 138-169 [$cPR3^{(138\text{-}169)}$] (NH_2-DLGWGVVGTHAAPAHGQALGAVGHWLVLLWQL-COOH) and the corresponding sense PR3 peptide [$PR3^{(138\text{-}169)}$] (NH_2-QLPQQDQPVPHGTQCLAM GWGRVGAHDPPAQV-COOH) were obtained from Alpha Diagnostic International (San Antonio, TX) with additional $cPR3^{(138\text{-}169)}$ peptide obtained from the University of North Carolina Peptide Synthesis Facility (Chapel Hill, NC). Chicken serum and yolk specific to $cPR3^{(138\text{-}169)}$ were obtained from Alpha Diagnostic. Rabbit serum specific to $cPR3^{(138\text{-}169)}$ was produced in house. Proteinase 3 was obtained from Elastin Products (Owensville, MO); plasminogen was obtained from Haematologic Technologies (Essex Junction, VT); elastase was obtained from Sigma (St. Louis, MO). We obtained rabbit antibody to histidine and goat antibody to plasminogen from Santa Cruz Biotech (Santa Cruz, CA); rabbit antisera to PR3 from Weislab AB (Lund, Sweden); alkaline phosphatase (AP)-conjugated goat antibody to human and rabbit IgG, AP-conjugated donkey antibody to goat IgG, AP-conjugated rabbit antibody to chicken IgY, horseradish peroxidase (HRP)-conjugated goat antibody to human and rabbit IgG, HRP-conjugated rabbit antibody to chicken IgY from Chemicon (Emecula, CA). Fluorescein isothiocyanate (FITC)-conjugated rabbit antibody to chicken and goat was obtained from Chemicon. The alkaline phosphatase substrate kit was obtained from Bio-Rad Laboratories (West Grove, PA). The SuperSignal chemiluminescent peroxidase substrate kit was obtained form Pierce Biotechnology (Rockland, IL). Western blots results were visualized by exposure to Biomax XAR film (Kodak, Syracuse, NY).

Recombinant complementary-PR3 protein production

We produced a recombinant, complementary-PR3 protein corresponding to PR3 residues 105-201 [$cPR3^{(105\text{-}201)}$] as previously described. Briefly, antisense PRTN3 DNA (nucleotides 166-456; GenBank accession no. X55668) was ligated to a BM40 secretion signal peptide and a 6x histidine tag and inserted into pcDNA3. Protein was expressed and secreted from HEK293 cells. Protein purification was performed using a HisTrap HP column (GE Healthcare, Piscataway, NJ). Cell supernate was applied to the HisTrap column, washed with 5 column volume's (CV) of binding buffer (PBS with 20 mM histidine, pH 7.6) and protein was removed from the column with 5 CV of elution buffer (PBS with 0.5 M histidine, pH 7.6). Protein elution was monitored by absorbance at 280 nm and verified by both ELISA and western blot using a rabbit anti-histidine antibody. The recombinant protein was dialyzed into phosphate-buffered saline (PBS) and the concentration was obtained with a protein assay using the Bio-Rad protein assay dye reagent and pre-aliquoted BSA standards.

*Affinity purification of anti-*cPR3$^{(138\text{-}169)}$ *antibody*

We affinity purified antibody specific to $cPR3^{(138\text{-}169)}$ for use in ELISA and western blot experiments using a $cPR3^{(138\text{-}169)}$ affinity column. $cPR3^{(138\text{-}169)}$ peptide was diluted to 1.0 mg/ml in DMSO. A HiTrap NHS-activated column (GE Healthcare) was washed with 6 ml of ice cold 1 mM HCl followed by addition of the $cPR3^{(138\text{-}169)}$ solution for 30 min at room temperature. The column was then washed and deactivated with 6 ml of alternating high pH (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3) and low pH (0.1 M acetate, 0.5 M NaCl, pH 4.0) buffer. The column was sealed for 30 min after the second addition of high pH buffer and sat

at room temperature, Washing finished with 6 ml each of low pH, high pH and low pH buffer. The column was stored in PBS (Invitrogen, Carlsbad, CA) with 0.1% NaN_3.

Antibodies specific for *cPR3*$^{(138\text{-}169)}$ peptide were affinity purified from immunized rabbit serum, chicken serum and chicken yolk. The solutions were first filtered through a 0.22 μm filter (Costar, Cambridge, MA) and then applied to the *cPR3*$^{(138\text{-}169)}$ column, the column was washed with 5 CV of PBS, and antibody was eluted with 0.1 M citric acid, pH 2.5. One ml aliquots were collected and antibody elution was monitored by absorbance at 280 nm. Antibody-containing fractions were neutralized immediately after elution with 100 μl of 1.0 M Tris, pH 9.0. The fractions were dialyzed into PBS overnight at 4°C.

Purification of proteins from plasmapheresis fluid

Use of human material was approved by the University of North Carolina-Chapel Hill Institutional Review Board and consent was obtained from all subjects. All column chromatography was performed on a AKTA fast performance liquid chromatography (FPLC) instrument (GE Healthcare) with a Frac-950 fraction collector (GE Healthcare). A 50 mL aliquot of plasmapheresis fluid (PLEX) was centrifuged at 3,000 x rpm to pellet insoluble material before filtering through a 0.22 μm filter (Costar). Total IgG was removed by passage of plasmapheresis fluid over a protein G column (GE Healthcare), washing the column with 5 CV of PBS, and eluting IgG with 0.1 M citric acid, pH 2.5 in 1 mL fractions. Elution of IgG was monitored by absorbance at 280 nm and fractions containing IgG were immediately neutralized by addition of 100 μL of 1.0 M Tris, pH 9.0. Total IgG was then dialyzed into PBS overnight at 4°C. To adsorb out as much IgG as possible, PLEX was typically passed over the protein G column a minimum of 3 times. No more IgG was

determined to be in the sample when the maximum protein absorbance during elution measured < 250 mAU. IgG-depleted PLEX was then concentrated 2-fold using ICON concentrators (Pierce, Rockford, IL). PLEX proteins were separated by passage of 3 mL aliquots over a Superdex 200 size exclusion column (GE Healthcare) and collection of 2 mL fractions. Protein elution was monitored by absorbance at 280 nm and protein-containing fractions were tested for reactivity to anti-*cPR3*$^{(138\text{-}169)}$ antibody by ELISA. Positive fractions by ELISA were pooled and further separated on a MonoQ ion exchange column (GE Healthcare). Samples were applied to the column in binding buffer (20 mM Tris, pH 8.0) and eluted by addition of a linear gradient of elution buffer (20 mM Tris, 0.5 M NaCl, pH 8.0).

*ELISAs showing PLEX proteins reactive to anti-*cPR3$^{(138\text{-}169)}$ *antibody*

High-binding 96-well plates (Costar) were coated with 50 µL of each protein-containing size exclusion fraction overnight at 4°C and blocked 1 hour in fish gelatin buffer (20 mM phosphate, 150 mM NaCl, 1% fish gelatin (Sigma), 0.05% Tween-20, pH 7.6). All subsequent steps were performed in blocking buffer and plates were washed 4 times with ELISA wash buffer (PBS plus 0.05% Tween-20) between each step. Wells were exposed to rabbit or chicken anti-*cPR3*$^{(138\text{-}169)}$ antibody (diluted 1:250) for 2 hours at room temperature. Proteins reactive to the anti-*cPR3*$^{(138\text{-}169)}$ antibodies were detected after addition of alkaline phosphatase-conjugated species specific secondary antibody for 1 hour (diluted 1:5000) at room temperature followed by addition of alkaline phosphatase substrate. Optical density at 405 nm was measured every 15 minutes for 2 hours using a VERSAmax tunable microplate

reader (Molecular Devices, Sunnyvale, CA). Positive fractions were determined visually by plotting absorbance versus fraction number.

ELISAs to test patient reactivity to cPR3$^{(105\text{-}201)}$

High-binding 96-well plates (Costar) were coated overnight at 4°C with *cPR3*$^{(105\text{-}201)}$ (5 μg/ml). Plates were blocked by addition of PBS with 1% goat serum (Sigma) for 1 hour at room temperature. All subsequent steps were performed in blocking buffer and plates were washed 4 times with ELISA wash buffer (PBS plus 0.05% Tween-20) between each step. Sera (diluted 1:100 in blocking buffer) were added for 2 hours at room temperature followed by AP-conjugated goat antibody to human IgG (diluted 1:10,000) for 1 hour. Optical density (OD) at 405 nm was measured after alkaline phosphatase substrate addition using a VERSAmax tunable microsplate reader. Rabbit antibody to histidine (diluted 1:100) served as a positive control. Levels of anti-*cPR3*$^{(105\text{-}201)}$ antibody in sera were expressed as percent OD of sera to positive control. Sera were considered positive when values exceeded the mean plus 2 standard deviations of healthy control subjects.

Western blot analysis of patient plasmapheresis proteins

Patient PLEX fractions from the size exclusion column that were reactive with the anti-*cPR3*$^{(138\text{-}169)}$ antibody were further evaluated by western blot. Twenty-five microliters of each fraction were combined with 8 μl of 4x SDS loading buffer and separated by 4% stacking, 10% separating SDS-PAGE. Proteins were then transferred to nitrocellulose (Whatman, Dassel, Germany), dried for 30 minutes to cross-link the proteins, blocked with 10% Blotto (Bio-Rad), and probed with primary and HRP-conjugated secondary antibody.

Reactivity was visualized by addition of SuperSignal substrate and exposure of the blots to Biomax XAR film.

Protein identification by mass spectrometry

An aliquot of ELISA-positive fractions was separated by SDS-PAGE and stained with coomassie R-250 (0.1% coomassie R-250 (Bio-Rad), 10% acetic acid (Fisher Scientific, Pittsburgh, PA). Protein bands reactive with anti-*cPR3*$^{(138\text{-}169)}$ antibody by western blot were then excised from the gel and subjected to in-gel tryptic digest. The digested fragments were then applied to matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) and identification was performed by database searching of identified peptides to a theoretical tryptic digest of the Swiss-Prot database. Identification of peptides was confirmed by MS/MS analysis.

Proteolysis assay

PR-3 (3.7 μM) was incubated with plasminogen (0.4 μM) for 90 min at 37ºC in PBS. The reaction was stopped by addition of 4x reducing or non-reducing SDS loading buffer. The sample was separated out by SDS-PAGE, fixed with fixing solution (25% isopropanol, 10% acetic acid) and stained with coomassie R-250 (0.1% coomassie R-250, 10% acetic acid). Elastase (0.4 μM) was incubated with plasminogen as a positive control while plasminogen alone was used as a negative control.

Flow cytometry analysis of normal human leukocytes

We determined whether the anti-*cPR3*$^{(138\text{-}169)}$ antibodies would react with normal leukocytes before attempting to identify putative complementary-PR3 proteins in patient cells. Fresh blood was obtained from healthy donors in Becton Dickinson vacutainer tubes K3 EDTA (purple top) (Franklin Lakes, NJ). Fifty microliters of blood was aliquoted to a 15 ml tube. Two ml of 1X lysing solution was added to each blood aliquot, the samples were mixed by shaking the tube and the samples then sat at room temperature for 10 min. The solution was spun at 500 x g for 5 min. The supernate was aspirated off while not disrupting the cell pellet, with approximately 100-200 µl of solution left over. Next, 500 µl 1X permeabilizing solution was added per tube, the tubes were vortexed briefly and they were left to sit at room temperature for 10 min. The cells were then pelleted by spinning at 500 x g for 5 min. The supernate was poured off, and the tubes were set upright with approximately 100 µl of solution left over. The chicken anti-*cPR3*$^{(138\text{-}169)}$ antibody was added so the final concentration was 0.1, 1 and 5 µg/ml. After addition of antibody, the samples sat at room temperature for 30 min. When the incubation was complete, 2 ml of wash buffer was added to each tube and pipetted up and down. The cells were then pelleted by spinning at 500 x g for 5 min. The supernate was poured off and 1 µl of FITC-conjugated rabbit antibody to chicken was added to each tube. The tubes were covered with aluminum foil and let sit at room temperature for 30 min. Two ml of wash buffer was added and the tubes were spun at 500 x g for 5 min. The supernate was poured off and 500 µl of 1% paraformaldehyde was added to each tube. The stained cells were analyzed using a FACscan flow cytometer linked to a CellQuest software system (Becton Dickinson Immunocytometry Systems, San Jose, CA).

RESULTS

A subset of PR3-ANCA patients have antibodies specific for cPR3$^{(105\text{-}201)}$

In order to determine in which patients to begin searching for complementary PR3 proteins, it was first necessary to determine which of the patients in the PR3-ANCA positive patient cohort had antibodies to a complementary PR3 protein. A recombinant, complementary PR3 protein corresponding to PR3 residues 105-201 [*cPR3*$^{(105\text{-}201)}$] was produced and tested against 72 PR3-ANCA patient sera for reactivity by ELISA. We found 13 of 72 (18%) PR3-ANCA patients tested positive, as defined by a value greater than two standard deviations above the mean of 63 healthy controls, to this recombinant *cPR3*$^{(105\text{-}201)}$ protein (Figure 3.1). Reactivity was expressed as OD of patient sera/OD of positive anti-His tag control. These results are similar to what was previously reported by our research group [49], even though in that study they examined a smaller group of PR3-ANCA positive patients.

*Plasminogen is a serum protein recognized by anti-*cPR3$^{(138\text{-}169)}$ *antibodies*

We produced rabbit and chicken polyclonal antibodies specific for a 30-mer section of the recombinant cPR3 (corresponding to residues 138-169 of PR3, *cPR3*$^{(138\text{-}169)}$) for use in identifying complementary PR3-like proteins *in vivo*. Rabbit antibodies specific for *cPR3*$^{(138\text{-}169)}$ were purified from total IgG while chicken antibodies specific for *cPR3*$^{(138\text{-}169)}$ were purified from serum and egg yolk with a *cPR3*$^{(138\text{-}169)}$ peptide affinity column.

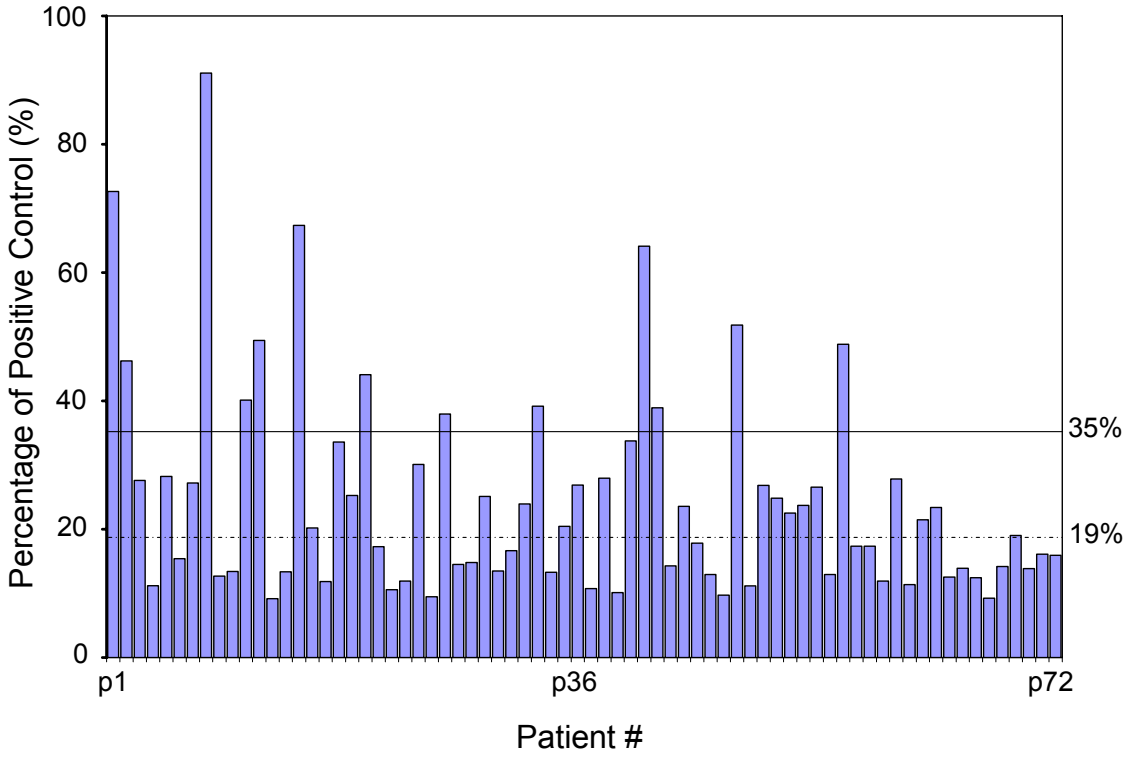

Figure 3.1. PR3-ANCA Patients React with *cPR3*$^{(105\text{-}201)}$.
PR3-ANCA patient (n = 72) and healthy control (n = 63) sera were screened for reactivity to a complementary PR3 protein corresponding to PR3 residues 105-201 [*cPR3*$^{(105\text{-}201)}$]. Data plotted is OD as a percentage of a positive control rabbit antibody to histidine. The mean value of the healthy controls is 19% (dashed line). All values above the mean plus two standard deviations of the healthy controls are considered positive (35%, solid line). A total of 13 of 72 (18%) patients were positive, compared with 6 of 63 (9.5%) healthy controls.

We searched for complementary PR3 proteins in plasmapheresis fluid, from a patient who tested positive for anti-*cPR3*$^{(105\text{-}201)}$ antibodies, by performing a series of chromatographic separations. The purification scheme was as follows: (1) IgG removal using a protein G affinity column, (2) size-exclusion chromatography of IgG-depleted plasmapheresis fluid, and (3) MonoQ ion-exchange chromatography of fractions that were reactive with the anti-*cPR3*$^{(138\text{-}169)}$ antibodies. We tested the size exclusion fractions for complementary PR3-like proteins by ELISA using the rabbit and chicken *cPR3*$^{(138\text{-}169)}$-specific antibodies. Figure 3.2A shows the peak of reactivity seen when PLEX fractions were probed with rabbit antibody along with a western blot of those reactive fractions indicating that the rabbit antibody reacted with three separate protein bands. Those fractions reactive with the rabbit antibody were then pooled and placed over a MonoQ ion exchange column. Figure 3.2B shows a coomassie stained gel of fractions off the MonoQ column with a corresponding western blot of the fractions. The reactive protein bands were sent to the UNC-Duke Michael Hooker Proteomics Facility for identification. The ~80 kDa protein band was identified as plasminogen, with the corresponding peptides identified by MS/MS inidicated in Figure 2.2B. The ~40 kDa band was identified as β_2-glycoprotein-1, with the corresponding identified peptides indicated. The middle protein band could not be accurately identified on the coomassie stained gel and thus its identity is unknown.

*Protein F from pseudomonas reacts with chicken anti-*cPR3$^{(138\text{-}169)}$ *antibodies*

In order to optimize our chances of identifying complementary PR3 proteins, and to confirm results obtained with the rabbit anti-*cPR3*$^{(138\text{-}169)}$ antibody, we produced anti-*cPR3*$^{(138\text{-}169)}$ antibodies in chicken. These antibodies were produced in two stages.

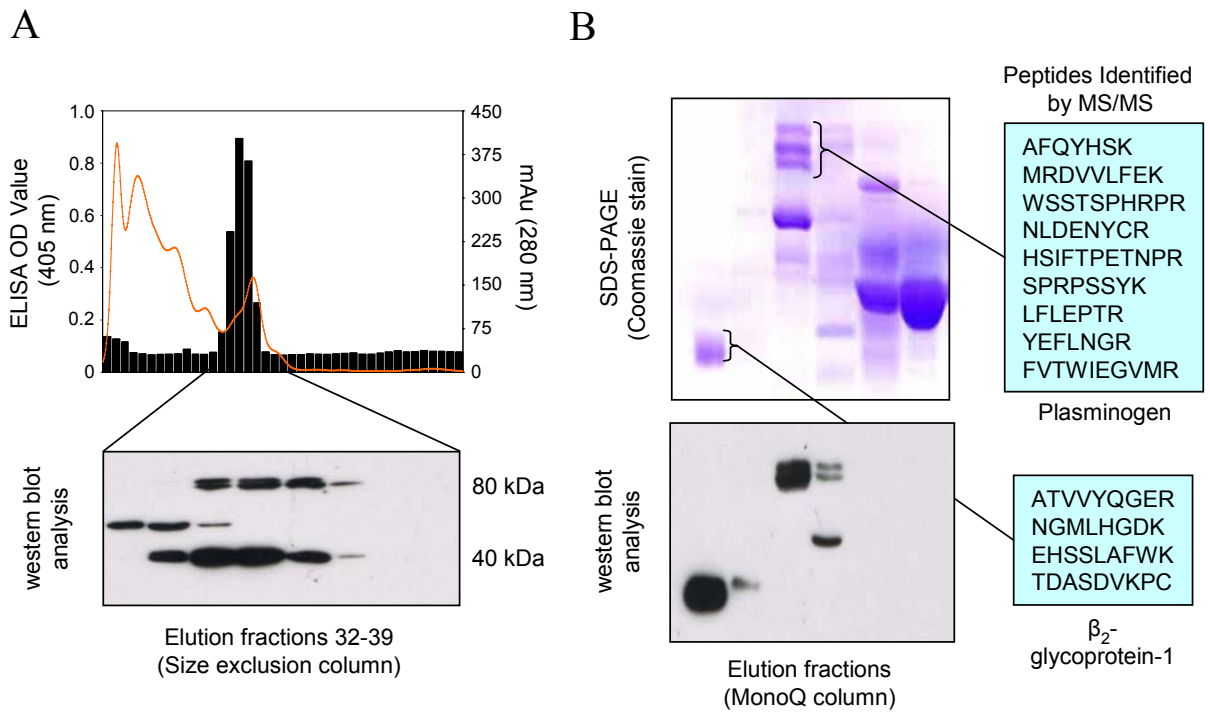

Figure 3.2. Identification of Complementary-PR3 Proteins Using Rabbit Anti-*cPR3*$^{(138-169)}$ Antibodies.

(A) Plasmapheresis proteins were fractionated by size exclusion chromatography and fractions 32-37 contained protein(s) reactive with rabbit anti-*cPR3*$^{(138-169)}$ antibodies by ELISA. Western blot analysis identified three reactive proteins (~80 kDa, ~50 kDa, ~40 kDa) using the rabbit antibody. (B) Fractions 32-37 from the size exclusion column were pooled and further purified on a MonoQ ion exchange column. The ~80, ~50 and ~40 kDa proteins were again eluted as determined by SDS-PAGE and western blot analysis. The ~80 kDa protein was identified as plasminogen by in-gel tryptic digest and mass fingerprinting while the ~40 kDa protein was identified as β2-glycoprotein-1. The ~50 kDa protein could not be identified on the coomassie stained gel.

Initially, the chicken was injected with *cPR3*$^{(138\text{-}169)}$ peptide and approximately 3 weeks post injection serum was isolated from the chicken and shipped to our laboratory. Anti-*cPR3*$^{(138\text{-}169)}$ antibodies were purified from the serum using a *cPR3*$^{(138\text{-}169)}$ affinity column. A second peptide injection was performed after serum isolation, and approximately 3 weeks post injection eggs were collected from the chicken and yolks were prepared by Alpha Diagnostic, Inc. and shipped to our laboratory. The yolk was passed over the *cPR3*$^{(138\text{-}169)}$ peptide column and anti-*cPR3*$^{(138\text{-}169)}$ antibodies were isolated.

The same size exclusion fractions that were tested by ELISA with the rabbit anti-*cPR3*$^{(138\text{-}169)}$ antibodies were then tested using chicken anti-*cPR3*$^{(138\text{-}169)}$ antibodies purified from serum after the first injection of *cPR3*$^{(138\text{-}169)}$ peptide into the chicken. Figure 3.3A shows the reactivity by ELISA seen with the serum-purified chicken antibody. Only one fraction appeared to contain protein(s) reactive to the chicken antibody, and when this fraction was evaluated by western blot there were reactive protein bands (Figure 3.3B). The ~26 kDa protein band was the only band visible by coomassie staining, and it was identified by mass spectrometry as Protein F from two different strains of Pseudomonas (Table 3.1).

Plasmapheresis proteins were separated by size exclusion chromatography from an additional PR3-ANCA patient as well as a non-ANCA nephropathy patient to determine if Protein F could be identified in either of those two samples. Figure 3.3C shows that the reactive protein band corresponding to Protein F is seen in the same fractions for both the additional PR3-ANCA patient tested as well as the non-ANCA nephropathy patient.

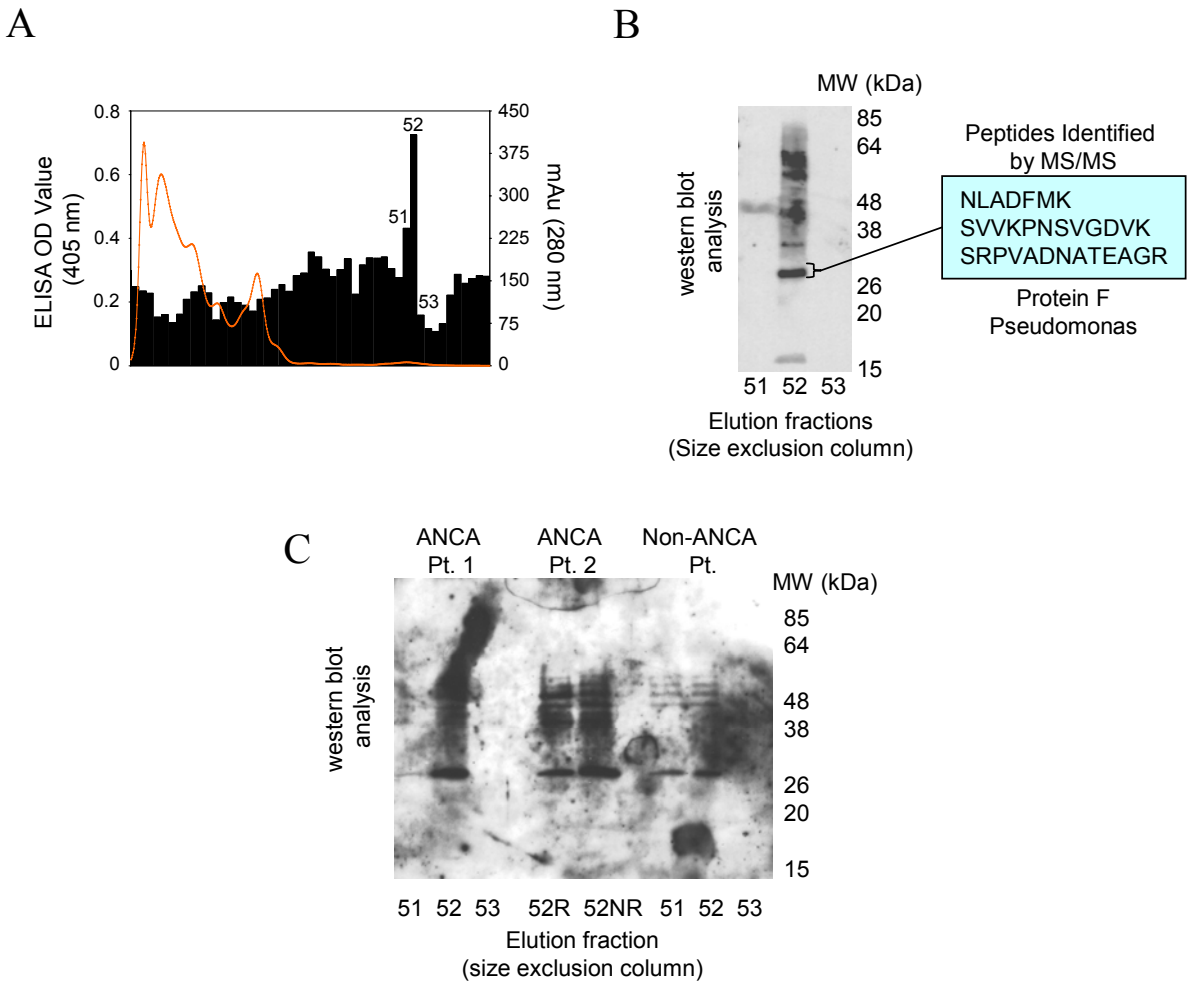

Figure 3.3. Identification of Complementary-PR3 Proteins Using Chicken Anti-*cPR3*$^{(138\text{-}169)}$ Antibodies from Serum.
(A) Plasmapheresis proteins were fractionated by size exclusion chromatography and fraction 52 contained protein(s) reactive with chicken anti-*cPR3*$^{(138\text{-}169)}$ antibodies by ELISA. (B) Western blot analysis identified multiple reactive proteins using the chicken antibody in fraction 52. The only band identifiable by coomassie staining was the ~26 kDa protein band, which was subsequently identified by mass spectrometry as Protein F from two separate Pseudomonas strains (P. tolaasii, P. fluorescens). The peptides identified in the Pseudomonas tolaasii protein are indicated in the blox. (C) Fractions from the size exclusion column were evaluated by western blot for two different ANCA patients and a non-ANCA nephropathy patient. Fraction 52 was evaluated under both reducing and non-reducing conditions for ANCA patient 2. The Protein F band is seen in samples from both ANCA patients as well as the nephropathy patient.

Table 3.1. Identification of Protein F from Pseudomonas.

RANK	Protein Name	Species	Accession No.	Protein MW	Peptide Count	Protein Score	Total Ion Score
1	Protein F precursor (Fragment).- Pseudomonas tolaasii.	Pseudomonas tolaasii	Q52465_PSETO	33,599.8	3	133	105

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification
854.4076	854.4138	0.0062	7	235	241	NLADFMK	2	0	Oxidation (M)[6]
854.4076	854.4138	0.0062	7	235	241	NLADFMK			
1292.6844	1292.6826	-0.0018	-1	223	234	SVVKPNSYGDVK	54	99.412	
1292.6844	1292.6826	-0.0018	-1	223	234	SVVKPNSYGDVK			
1343.6663	1343.6672	0.0009	1	296	308	SRPVADNATEAGR	51	98.895	
1343.6663	1343.6672	0.0009	1	296	308	SRPVADNATEAGR			

RANK	Protein Name	Species	Accession No.	Protein MW	Peptide Count	Protein Score	Total Ion Score
2	Protein F precursor (Fragment).- Pseudomonas fluorescens	Pseudomonas fluorescens	Q51780_PSEFL	33,414.6	2	105	91

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification
1141.5344	1141.536	0.0016	1	120	130	TNVQADGHSGR	37	70.192	
1141.5344	1141.536	0.0016	1	120	130	TNVQADGHSGR			
1292.6844	1292.6826	-0.0018	-1	223	234	SVVKPNSYGDVK	54	99.412	
1292.6844	1292.6826	-0.0018	-1	223	234	SVVKPNSYGDVK			

Chicken anti-cPR3$^{(138\text{-}169)}$ *antibodies purified from yolk react with plasminogen*

Size exclusion fractions were also tested using chicken anti-*cPR3*$^{(138\text{-}169)}$ antibodies purified from egg yolk obtained after a second injection of *cPR3*$^{(138\text{-}169)}$ peptide into the chicken (Figure 3.4A). Multiple reactive fractions were identified by ELISA, however when these fractions were pooled and analyzed by western blot, only one protein band was detected (Figure 3.4B). This reactive protein band from pooled fractions group 1 was sent to mass spectrometry and was identified as a plasminogen fragment. The fraction containing Protein F was not reactive with this anti-*cPR3*$^{(138\text{-}169)}$ antibody preparation.

PR3 and plasminogen are complementary proteins

Complementary proteins, as defined by Root-Bernstein, are theorized to interact and to give rise to idiotypic antibodies [92]. Since plasminogen and PR3 are not complementary proteins as defined by Mekler, that is they are not encoded by sense-antisense codons, we attempted to determine if PR3 and plasminogen fit the Root-Bernstein definition of complementary proteins. If PR3 and plasminogen bind together, an expected result would be that PR3 would cleave plasminogen, based on a report that elastase, a close homolog of PR3, cleaves plasminogen [130]. Addition of PR3 to plasminogen resulted in cleavage of plasminogen (Figure 3.5A) indicating that these two proteins physically interact. Elastase cleaves plasminogen to the anti-angiogenesis compound angiostatin, and PR3 derived fragments of plasminogen were similar in size to the elastase derived fragments, although the exact location of PR3 cleavage on plasminogen was not determined.

A second expected result if plasminogen and PR3 are complementary proteins would be that production of antibodies to one of the proteins would lead to production of antibodies

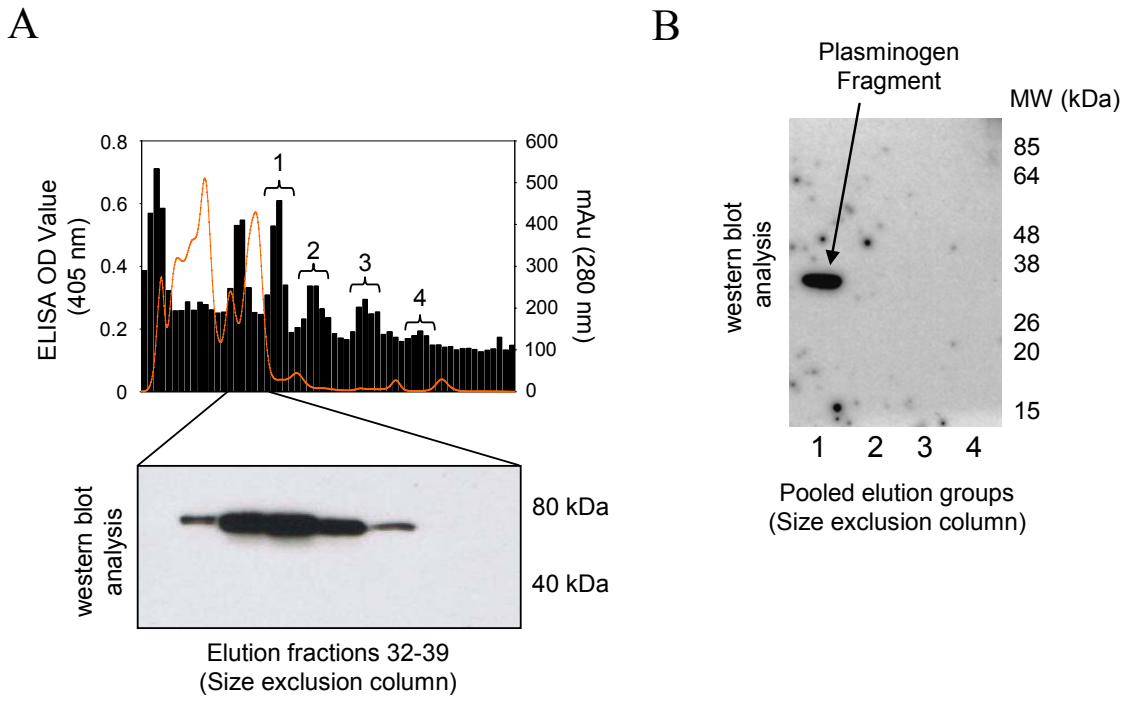

Figure 3.4. Identification of a Complementary-PR3 Protein Using Chicken Anti-*cPR3*$^{(138\text{-}169)}$ Antibodies from Egg Yolk.

(A) Plasmapheresis proteins were fractionated by size exclusion chromatography and multiple fraction contained protein(s) reactive with chicken anti-*cPR3*$^{(138\text{-}169)}$ antibodies by ELISA. (B) Western blot analysis of the pooled elution fractions identified a reactive protein band in pooled fraction 1, while no other reactive proteins were detected. The reactive protein band was identified as a plasminogen fragment by mass spectrometry.

against the other protein through the idiotypic netowork. Our research group had previously shown that injection of *cPR3*$^{(138\text{-}169)}$ into mice resulted in the production of antibodies to not only *cPR3*$^{(138\text{-}169)}$ but also to PR3. We decided to test this model in the opposite direction, i.e. would injection of PR3 result in production of antibodies to plasminogen? Plasmapheresis fractions were probed for the presence of PR3 using serum from a rabbit immunized with human PR3. PR3 was not detected, however, the rabbit PR3-specific serum contained antibodies reactive with plasminogen (Figure 3.5B), in contrast to pre-immune serum that was non-reactive with both PR3 and plasminogen. Presumably, these anti-plasminogen antibodies were made through the idiotypic network as the rabbit had no exposure to human plasminogen.

Lastly, we examined whether an intact idiotypic network was active in chicken immunized with *cPR3*$^{(138\text{-}169)}$. We were able to isolate anti-PR3 antibodies from the same chicken immunized with *cPR3*$^{(138\text{-}169)}$ peptide using the corresponding PR3 sense peptide [*PR3*$^{(138\text{-}169)}$]. We tested these antibodies' reactivity to both *cPR3*$^{(105\text{-}201)}$ and PR3. Anti-*cPR3*$^{(138\text{-}169)}$ antibodies, previously shown to react to plasminogen, bind to *cPR3*$^{(105\text{-}201)}$ but not with PR3 (Figure 3.5C). Anti-*PR3*$^{(138\text{-}169)}$ antibodies purified from the *cPR3*$^{(138\text{-}169)}$ immunized chicken react with PR3 but not with *cPR3*$^{(105\text{-}201)}$. This further confirms that the anti-*cPR3*/anti-plasminogen antibodies are a separate pool of antibodies from the anti-PR3 antibodies. In addition, since anti-*cPR3*$^{(105\text{-}201)}$ antibodies have previously been shown to be the idiotypic partner to anti-PR3 antibodies, it means that anti-PR3 and anti-plasminogen antibodies are idiotypic.

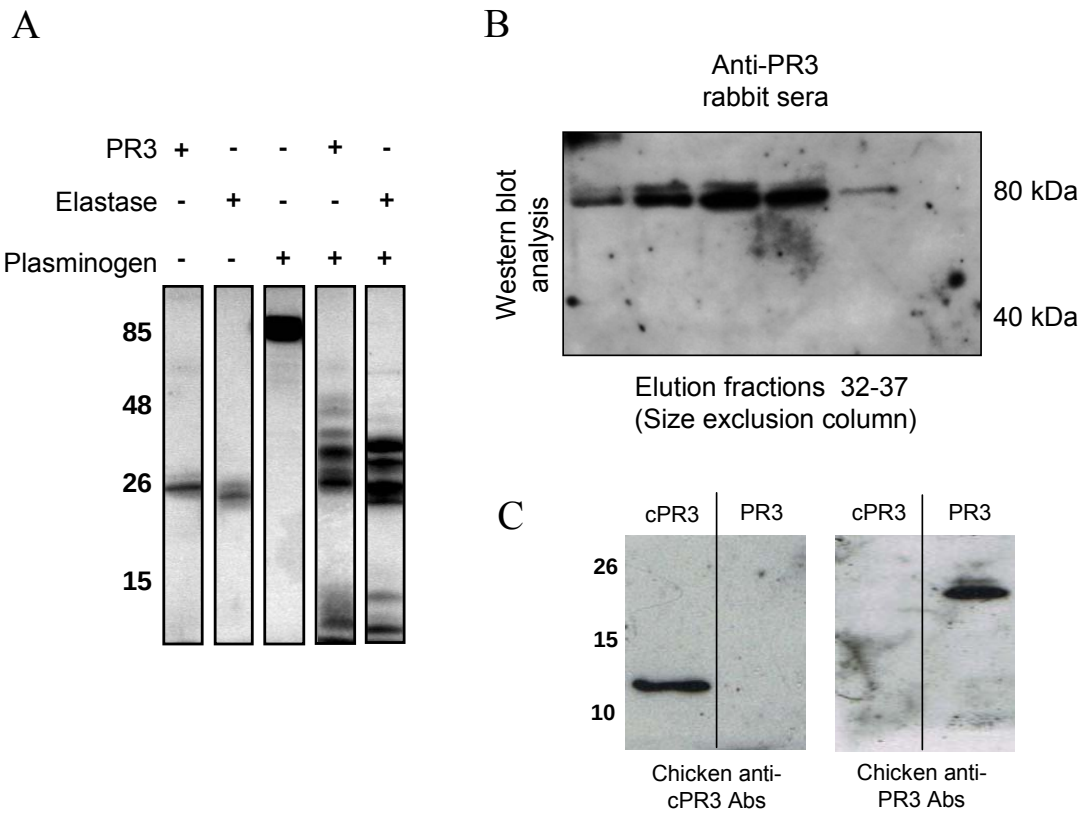

Figure 3.5. Plasminogen and PR3 are Complementary Proteins.
(A) Plasminogen was incubated with PR3 or elastase (known to cleave plasminogen to the anti-angiogenesis compound angiostatin) for 1 hour at 37°C. The reaction was stopped by addition of non-reducing SDS sample buffer, the samples were separated by SDS-PAGE, and stained with coomassie R-250. The results show cleavage of plasminogen by native PR3. (B) To determine if native PR3 was present in fractions from the size exclusion column, a western blot was performed utilizing serum from a rabbit immunized with human PR3. The 30 kDa PR3 protein band was not present in any fraction, however there was reactivity with the 80 kDa band identified as plasminogen. (C) The idiotypic network is active in chicken immunized with *cPR3*$^{(138\text{-}169)}$ peptide. Chicken anti-*cPR3*$^{(138\text{-}169)}$ antibodies, purified using a *cPR3*$^{(138\text{-}169)}$ peptide columan and shown previously to react with plasminogen, react with *cPR3*$^{(105\text{-}201)}$ but not with PR3. Anti-PR3 antibodies were purified from the same chicken, using a *PR3*$^{(138\text{-}169)}$ sense peptide, and these antibodies react with PR3 but not with *cPR3*$^{(105\text{-}201)}$ or plasminogen.

Anti-cPR3$^{(138\text{-}169)}$ *antibodies bind to normal human leukocytes*

We attempted to search patient leukocytes for additional complementary-PR3 proteins using our anti-*cPR3*$^{(138\text{-}169)}$ antibodies. Before doing so, we determined if the antibodies would bind to normal leukocytes. We isolated leukocytes from healthy donors, fixed and permeabilized the cells and then stained them with anti-*cPR3*$^{(138\text{-}169)}$ antibody isolated from chicken egg yolk before analyzing them by flow cytometry. In addition, we stained the cells with anti-CD14 and anti-CD3 so that we could positively identify the monocytes and lymphocytes, respectively (Figure 3.6A). The results show that the anti-*cPR3*$^{(138\text{-}169)}$ antibody stains all leukocytes (Figure 3.6B). These results were seen for two different concentrations of anti-*cPR3*$^{(138\text{-}169)}$ antibody. Normal chicken IgY did not stain any of the cells. After a literature search we discovered that plasminogen is known to bind to all leukocytes with relatively high affinity [131].

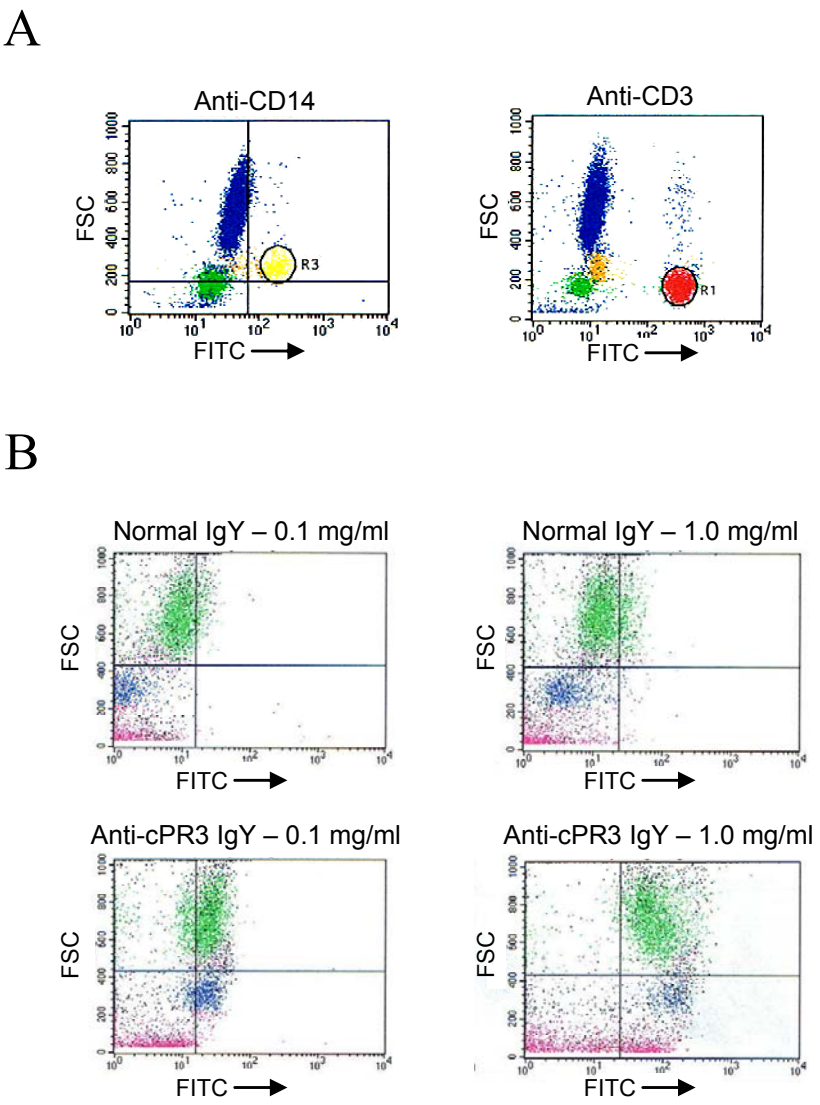

Figure 3.6. Anti-*cPR3*$^{(138\text{-}169)}$ Antibodies Bind to Normal Human Leukocytes.
(A) Normal human leukocytes were fixed and permeabilized before analysis by flow cytometry. Cells were initially stained with anti-CD14 and anti-CD3 so that monocytes and lymphocytes could be positively identified. Neutrophils and other granulocytes make up the group of cells colored in blue. (B) Normal human leukocytes were stained with normal chicken IgY or anti-*cPR3*$^{(138\text{-}169)}$ at two different concentrations. Chicken anti-*cPR3*$^{(138\text{-}169)}$ antibodies stain all leukocytes, while normal chicken IgY and goat IgG do not stain.

DISCUSSION

By capitalizing on complementary protein-protein interactions, based on the theory of autoantigen complementarity, we searched for proteins complementary to a known human autoantigen, PR3. We began by identifying those patients who had circulating antibodies to a recombinant, complementary PR3 protein [$cPR3^{(105-201)}$]. The fact that 18% of the PR3-ANCA patients tested were positive for these antibodies is encouraging and supports the hypothesis put forth in the theory of autoantigen complementarity that a previous exposure to a complementary protein could have initiated the production of PR3-ANCA. Previously, our research group had published that 7/34 (20%) PR3-ANCA patients tested positive for antibodies to $cPR3^{(105-201)}$, thus the results seen in this study were very comparable.

Why do some PR3-ANCA patients have antibodies to a complementary-PR3 protein? There are at least two explanations available. The first is that patients are in fact exposed to some type of complementary protein, be it produced endogenously or brought in exogenously, that results in antibody production and subsequent anti-idiotypic antibody production leading to an autoimmune reaction. The second explanation is that these antibodies that react with the complementary protein are simply produced as a result of the idiotypic network and in fact are secondary to the production of PR3-ANCA. As discussed in the Prologue, there are reports of anti-idiotypic antibodies from a host of autoimmune diseases, including SLE, MPO-ANCA vasculitis, MG and diabetes. Smith et al showed that idiotypic antibodies are produced by complementary peptides, thus even if these anti-complementary protein antibodies were produced secondarily to the PR3-ANCA it isn't surprising that they would react to a contrived, complementary protein. Alternatively, the presence of anti-idiotypic antibodies in those other autoimmune diseases could be support for

a wider application of the theory of autoantigen complementarity than just to ANCA vasculitis. Since we only have serum available from patients after their diagnosis, it is nearly impossible to determine which antibody response came first. Other investigators have utilized the United States Department of Defense serum repository, which contains over 30 million serum samples collected over time from over 5 million armed forces members, to study when autoantibodies formed in those patients later diagnosed with an autoimmune disease. Arbuckle et al used samples from 130 individuals in that repository and showed that antibodies to SLE antigens form years before a diagnosis of SLE is made in those individuals [132]. A study similar to that could be performed to determine if anti-complementary protein antibodies arise before the presence of autoantibodies and development of disease.

We produced antibodies to *cPR3*$^{(138\text{-}169)}$ and using these antibodies we identified two putative, complementary-PR3 proteins in plasmapheresis fluid taken from two different PR3-ANCA patients. The discovery of these two proteins, plasminogen and Protein F, was surprising given that plasminogen is a ubiquitous protein with a prominent role in fibrinolysis in all individuals and Protein F is a bacterial protein that you would typically not expect to find circulating in human serum. Neither of these proteins is encoded by the antisense strand of the PR3 gene and neither of these proteins has significant sequence similarity to *cPR3*$^{(105\text{-}201)}$, thus they do not fit neatly into the theory of autoantigen complementarity. However, given that an antibody raised to a complementary PR3 protein reacts with both of them means that there is some relationship present between those proteins and PR3.

How does this theory of autoantigen complementarity work, and how can it be utilized to further discover initiating antigens in autoimmune diseases? The presence of complementary proteins has long been described. Mekler noted in the 1960's that proteins

translated off the sense and anti-sense RNA strands in the same reading frame interacted like complementary nucleic acid strands. Complementary protein pairs have been extensively studied and mostly been used to investigate receptor-ligand interactions for biochemical engineering studies. There is no clear explanation for why complementary proteins interact, although Blalock et al. have proposed that it is complementary shape, brought about by the inverse hydropathic profiles of complementary proteins, that drives binding. They have furthered our understanding of complementary proteins and their relationship to the immune system by demonstrating that antibodies raised to complementary peptides bound in an idiotypic fashion, i.e. through their variable domains. This relationship was similar to what had previously been demonstrated with PR3-ANCA and antibodies to complementary-PR3 protein,

Root-Bernstein has posited a variation on these themes, defining a complementary pair of proteins as capable of stereospecific binding and induction of molecularly complementary antibodies or T-cell antigen receptors. In this model, two molecularly complementary antigens bind together and are processed by the immune system simultaneously. Tolerance is broken if one or both of those molecularly complementary antigens has homology with a "self" protein. PR3 and plasminogen bind to each other, as expected for a complementary pair, and plasminogen is a substrate for PR3, which documents physical interaction. We show here that a rabbit immunized with human PR3 developed antibodies not only to PR3 but also to plasminogen, presumably through the idiotypic network as pre-immune serum from the rabbit did not react with either PR3 or plasminogen. Mice, rabbits and chicken (which we show in this research) that were inoculated with complementary-PR3 peptide developed antibodies to this peptide as well as

PR3, suggesting that the idiotypic network is responsible for the derivation of the secondary antibody response, not necessarily the binding of proteins together. However, binding of these complementary pairs to each other may augment antigenic propensity. It appears that PR3 and plasminogen are complementary proteins as defined by Root-Bernstein, thus utilizing that definition of complementarity is likely called for as it pertains to the theory of autoantigen complementarity. However, the theory of antigenic complementarity is not necessarily correct, as we have shown that anti-idiotypic antibodies can form without the presence of a second antigen.

Now that PR3 and plasminogen are known to bind, and antibodies to PR3 and plasminogen are idiotypic, it would be interesting to determine the interface where PR3 and plasminogen interact. The reason for this is to test whether the surfaces involved in PR3/plasminogen binding are the same that bind to their respective antibodies. Proving this would likely open an entirely new avenue of thinking in regards to protein-protein interactions and protein-antibody interactions. Jerne hypothesized that the anti-idiotypic antibodies would hold the "internal image" of the original antigen. In the case of PR3 and plasminogen that would mean PR3-ANCA would bind to the same region of PR3 that plasminogen binds to, and vice versa. To prove this, it would be necessary to either 1) show where PR3 cleaves plasminogen or 2) resolve a crystal structure of plasminogen and PR3 in complex (using a non-enzymatically active form of PR3). However, this may not necessarily be the only interaction between the two proteins. It is possible that PR3 could bind to a portion of plasminogen and then cleave at an entirely separate region. A crystal structure of the two proteins bound together could help in this matter. Another set of data that would prove extremely helpful would be identification of epitopes on PR3 recognized by PR3-

ANCA. Thus far, only PR3-peptide binding studies have been employed to determine where on PR3 the ANCA bind. These studies have not given a definitive answer, other than there are multiple epitopes recognized by ANCA, both within a single patient and within a population of patients. We did attempt one round of epitope mapping using affinity purified PR3-ANCA and the method described utilizing mass spectrometry. The results of that experiment were inconclusive as the only epitope identified by mass spectrometry was also seen when a control IgG was incubated with PR3. Since that method does have limitations, and since PR3-ANCA are known to bind to conformational epitopes, it will prove very difficult to epitope map those autoantibodies.

Another idea to consider with regard to multiple epitopes recognized by PR3-ANCA is that there could be additional autoantibody pools in PR3-ANCA patients that recognize different PR3 binding proteins. Identification of a series of PR3 binding proteins could be used in an ELISA to determine if PR3-ANCA patients had autoantibodies to any of those proteins. If other such autoantibodies were found, it would be very interesting to compare the PR3-ANCA epitope mapping data with other autoantibodies the patients have and determine if there is any correlation, i.e. if ANCA bind to a certain epitope on PR3 do those patients more often than not have anti-plasminogen autoantibodies.

Identification of Protein F in plasmapheresis fluid from two ANCA patients and a non-ANCA patient is certainly intriguing. The strains of pseudomonas that Protein F is found are not pathogenic. One strain, P. tolaasii is a mushroom pathogen while the other strain, P. fluorescens is part of the normal flora of the large intestine. Protein F from those two strains is very similar, with close to 90% homology between the two. While only three peptides of Protein F were identified in the mass fingerprinting, the scores generated were

still considered significant. Protein F, like plasminogen, shares little to no sequence homology with *cPR3*$^{(138\text{-}169)}$, thus it is difficult to determine how and why these proteins were identified by antibodies raised to *cPR3*$^{(138\text{-}169)}$. PR3-ANCA patients typically complain of suffering from "flu-like" symptoms shortly before being diagnosed with ANCA disease, thus a microbial protein leading to the onset of PR3-ANCA is certainly possible. While we did not pursue the Protein F story further for this project, clearly this will need to be followed up on. Cloning of protein F from one or both strains of pseudomonas would allow an ELISA screening of patient sera to determine if any PR3-ANCA patients have anti-Protein F antibodies. The results of that experiment would then help determine if the identification of Protein F by anti-*cPR3*$^{(138\text{-}169)}$ antibodies was real or simply an artifact.

These studies were designed to explore if the theory of autoantigen complementarity could lead to the detection of a novel autoimmune response. The discovery that plasminogen is complementary to PR3 provides additional support for the theory of autoantigen complementarity.

CHAPTER IV

Identification and Characterization of Anti-plasminogen Autoantibodies in PR3-ANCA Positive Vasculitis Patients

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ABSTRACT

PR3-ANCA vasculitic autoimmune disease is characterized by autoantibodies directed agasint the neutrophil protein PR3. Previous results showed that an antibody raised to a recombinant, complementary protein expressed off the antisense strand of the PR3 gene recognized plasminogen from PR3-ANCA patient plasmapheresis fluid. The current study demonstrates that a subset of PR3-ANCA patients have antibodies directed against plasminogen that have a functional consequence. Anti-plasminogen antibodies were purified from two separate PR3-ANCA patients using a $cPR3^{(138\text{-}169)}$ affinity column. Antibody epitopes were determined by degradation of non-epitope regions followed by mass spectrometry of protected fragments. Fibrin clot assays measured the functional effects of these autoantibodies. Serologic studies searching for antibodies to plasminogen were assayed by ELISA in 106 ANCA SVV patients, 57 patients with idiopathic thrombosis and 63 healthy controls. Anti-plasminogen autoantibodies recognized a surface-exposed structure within plasminogen's protease domain, effectively decreased the conversion of plasminogen to plasmin and delayed clot dissolution *in vitro*. Anti-plasmiongen autoantibodies were identified in a subset of PR3-ANCA patients but not controls and these patients had increased risk for thrombotic events. This is the first example in human disease of a complementary protein pair (PR3 and plasminogen) giving rise to complementary antibodies where one of those antibodies also correlates with a clinical complication.

INTRODUCTION

A number of prevailing theories on the origin of human autoimmunity provide insights into disease causation [133]. These approaches have been difficult to translate into curative therapy in human autoimmune disease and, as a consequence, most therapy is aimed at suppressing the inflammatory responses.

In patients who have ANCA SVV, autoantibodies react with neutrophils and monocytes causing aberrant activation and subsequent vascular injury. There are increasing data implicating complementary proteins in autoantibody production (discussed earlier in Chapter 1). Complementary protein pairs have a natural affinity for binding and are derived from a number of sources, including the transcription and translation of complementary strands of DNA [99]. The phenomenon of complementarity is proposed to be a driving force in autoimmune responses through a number of mechanisms. This concept became more likely with the demonstration that patients with PR3-ANCA SVV harbor antibodies against proteins complementary to the corresponding autoantigen, PR3. Demonstration of idiotypic pairing of these two coexisting antibodies led to the theory of autoantigen complementarity. Antibodies raised in rabbits and chicken to the complementary PR3 protein recognized plasminogen. Now the question arises as to whether patients have antibodies to plasminogen. In the current study, anti-plasminogen autoantibodies were identified in a subset of PR3-ANCA patients, they delayed fibrin clot dissolution *in vitro* and, of clinical significance, occurred most commonly in PR3-ANCA patients with coincident thrombotic events.

MATERIALS AND METHODS

Study Population

The 106 patients with ANCA SVV in this study had pauci-immune necrotizing and crescentic glomerulonephritis and a positive PR3-ANCA (n = 72) or myeloperoxidase (MPO)-ANCA (n = 34) determination. Patients were classified into types of ANCA-SVV as defined by the Chapel Hill nomenclature [18]. Patients consented to long-term follow up in the prospective cohort studies from the time of disease onset (diagnostic entry biopsy) until death. Blood samples were available at the time of clinically indicated diagnostic vasculitis testing and thus not always available at the time of venous thrombotic events.

A clotting control group was composed of 57 patients randomly selected from the thrombophilia service with a history of idiopathic deep vein thrombosis or pulmonary emboli of unknown etiology. All study participants were evaluated for the presence of classic risk factors for venous thromboembolism. A group of 63 healthy individuals who were either kidney or blood donors constituted a healthy control group. All studies were approved by the University of North Carolina School of Medicine Institutional Review Board.

Antigens/Antibodies/Reagents

Complementary-PR3 peptide corresponding to PR3 residues 138-169 [$cPR3^{(138\text{-}169)}$] (NH_2-DLGWGVVGTHAAPAHGQALGAVGHWLVLLWQL-COOH) was obtained from Alpha Diagnostic International (San Antonio, TX) and the University of North Carolina Peptide Synthesis Facility (Chapel Hill, NC). The particular amino acid sequence of cPR3 peptide used in this study, amino acids 138-169, was first identified as an epitope of patients'

antibodies during a screen of a bacterial expression library [49]. Plasminogen, plasmin and thrombin were obtained from Haematologic Technologies (Essex Junction, VT); tissue-type plasminogen activator (tPA) was graciously provided by Dr. Alisa Wolberg; urokinase-type plasminogen activator (uPA) was obtained from Sigma (St. Louis, MO). Spectrozyme PL was obtained from American Diagnostica (Stamford, CT). We obtained rabbit antibody to histidine and goat antibody to plasminogen from Santa Cruz Biotech (Santa Cruz, CA); normal human IgG from Bethyl Laboratories (Montgomery, TX); alkaline phosphatase (AP)-conjugated goat antibody to human and rabbit IgG, AP-conjugated donkey antibody to goat IgG, horseradish peroxidase (HRP)-conjugated goat antibody to human IgG from Chemicon (Emecula, CA). The alkaline phosphatase substrate kit was obtained from Bio-Rad Laboratories (West Grove, PA). The SuperSignal chemiluminescent peroxidase substrate kit was obtained form Pierce Biotechnology (Rockland, IL). Western blots results were visualized by exposure to Biomax XAR film (Kodak, Syracuse, NY).

*Affinity purification of patient anti-*cPR3$^{(138\text{-}169)}$ *antibody*

We affinity purified antibody specific to *cPR3*$^{(138\text{-}169)}$ for use in ELISA and western blot experiments using a *cPR3*$^{(138\text{-}169)}$ affinity column. Preparation of the *cPR3*$^{(138\text{-}169)}$ affinity column was discussed in Chapter III. Total human IgG isolated from PR3-ANCA patient PLEX was filtered through a 0.22 μm filter (Costar, Cambridge, MA) and then applied to the *cPR3*$^{(138\text{-}169)}$ column, the column was washed with 5 column volumes (CV) of PBS, and antibody was eluted with 0.1 M citric acid, pH 2.5. One ml aliquots were collected and antibody elution was monitored by absorbance at 280 nm. Antibody-containing fractions

were neutralized immediately after elution with 100 µl of 1.0 M Tris, pH 9.0. The fractions were dialyzed into PBS overnight at 4°C.

*Western blot analysis of patient anti-*cPR3$^{(138\text{-}169)}$ *reactivity*

To determine if patient affinity purified anti-*cPR3*$^{(138\text{-}169)}$ antibodies were reactive with plasminogen (as are rabbit and chicken anti-*cPR3*$^{(138\text{-}169)}$ antibodies, see Chapter III) the patient antibodies were used for western blot analysis of elution fractions. Twenty-five microliters of each PLEX fraction were combined with 8 µl of 4x SDS loading buffer and separated by 4% stacking, 10% separating SDS-PAGE. Proteins were then transferred to nitrocellulose (Whatman, Dassel, Germany), dried for 30 minutes to cross-link the proteins, blocked with 10% Blotto (Bio-Rad), and probed with patient affinity purified anti-cPR3(138-169) antibody (1:100 dilution) and HRP-conjugated secondary antibody. Reactivity was visualized by addition of SuperSignal substrate and exposure of the blots to Biomax XAR film.

Patient anti-*cPR3*$^{(138\text{-}169)}$ antibody reactivity to plasminogen and plasmin was evaluated by western blot. One µg of plasminogen and plasmin were combined with 8 µl of 4x SDS non-reducing and reducing loading buffer and separated by 4% stacking, 10% separating SDS-PAGE. Proteins were then transferred to nitrocellulose (Whatman, Dassel, Germany), dried for 30 minutes to cross-link the proteins, blocked with 10% Blotto (Bio-Rad), and probed with patient affinity purified anti-*cPR3*$^{(138\text{-}169)}$ antibody (1:100 dilution) and HRP-conjugated secondary antibody. Reactivity was visualized by addition of SuperSignal substrate and exposure of the blots to Biomax XAR film.

Epitope mapping of anti-plasminogen autoantibodies

Epitope mapping of anti-plasminogen antibodies utilizing MALDI-MS was performed as a collaboration with the UNC-Duke Michael Hooker Proteomics Facility as previously described [134]. Briefly, affinity purified anti-*cPR3*$^{(138\text{-}169)}$ antibody or PR3-ANCA patient total IgG was covalently linked to CNBr-activated sepharose beads (GE Healthcare) according to the manufacturer's instructions. The antibody-linked beads were incubated with 50 µg plasminogen or *cPR3*$^{(138\text{-}169)}$ peptide for 2 hours at room temperature and submitted for analysis.

A series of proteolytic digestions is used to cleave protein segments that are not protected by the antibody, and thus not part of the epitope. The beads are then washed and the MALDI matrix breaks apart the epitope/antibody interaction, freeing the peptide fragment. This fragment is then subjected to MALDI analysis to determine what portion of the protein is still bound to the antibody after each addition of protease. This procedure is successful as the antibodies themselves are rather resistant to proteolysis. A theoretical digestion can be performed for each addition of protease and this theoretical list of fragments can be compared to the MALDI mass spectrum to determine what fragments are still bound to antibody. The first protease used was Lys-C (Wako Chemicals, Richmond, VA), which cleaves after lysine residues and results in relatively large fragments to analyze. This was followed by addition of Trypsin-TPCK (Worthington Biochemical, Lakewood, NJ). Finally, N-terminal and C-terminal degradation is performed with Aminopeptidase M (Roche Applied Science, Indianapolis, IN) and Carboxypeptidase Y (Roche Applied Science). MS/MS analysis was performed to confirm peptide identity. Antibody with no protein added was utilized as a negative control.

In vitro *plasminogen assays*

To test the activity of the anti-plasminogen autoantibodies, a set of *in vitro* plasminogen assays was performed. Plasminogen (15 μg/mL final concentration) was pre-incubated with affinity purified patient anti-plasminogen autoantibodies (30 μg/mL final concetnration), control human total IgG (30 μg/mL final concentration) or HBS buffer (20 mM HEPES, 150 mM NaCl, 5 mM Ca^{2+} pH 7.4) for 10 min in a 1.5 ml eppendorf tube. uPA (3 nM final concentration) or tPA (13 μg/mL final concentration) was combined with Spectrozyme PL (500 μM final concentration) in HBS buffer and placed in a 96-well plate (Costar) and the plasminogen/antibody mixture was added to begin the assay. Change in absorbance at 405 nm was monitored in duplicate samples using a VERSAmax tunable microplate reader ((Molecular Devices, Sunnyvale, CA).

To determine the anti-fibrinolytic effects of the affinity-purified patient anti-plasminogen autoantibodies, normal human plasma (90% final concentration) was pre-incubated with affinity purified autoantibodies (50 μg/mL final concentration), control human total IgG (50 μg/mL final concentration) or HBS buffer for 10 min in a 1.5 ml eppendorf tube. Plasma was added to a 96-well plate (Costar) containing thrombin (15 nM final concentration) and uPA (30 nM final concentration). Clot formation and dissolution were monitored by absorbance at 405 nm using a VERSAmax tunable microplate reader. Two independent experiments were performed using affinity purified anti-plasminogen antibody from two different PR3-ANCA patients.

ELISAs to test patient reactivity to plasminogen

High-binding 96-well plates (Costar) were coated overnight at 4°C with plasminogen (5 μg/ml). Plates were blocked by addition of PBS with 1% goat serum (Sigma) for 1 hour at room temperature. All subsequent steps were performed in blocking buffer and plates were washed 4 times with ELISA wash buffer (PBS plus 0.05% Tween-20) between each step. Sera (diluted 1:100 in blocking buffer) were added for 2 hours at room temperature followed by AP-conjugated goat antibody to human IgG (diluted 1:50,000) for 1 hour. Optical density at 405 nm was measured after alkaline phosphatase substrate addition using a VERSAmax tunable microplate reader. Goat antibody to plasminogen (diluted 1:500) served as a positive control. Levels of anti-plasminogen antibody in sera were expressed as percent OD of sera to positive control. Sera were considered positive when values exceeded the mean plus 2 standard deviations of healthy control subjects.

Statistical Analysis

Statistical analysis of the *in vitro* plasminogen assays was performed using a Student's *t* test. Anti-plasminogen autoantibodies are plotted as a continuous measure of the percentage of positive control. However, the frequency of positive anti-plasminogen autoantibodies, and not the continuous value, was the primary measure of interest. Therefore, statistical comparisons between the prevalence of positives between patient groups were evaluated using a 2-sided Fisher's exact test to accommodate the small number of positive values in several patient groups.

RESULTS

Anti-cPR3$^{(138\text{-}169)}$ *antibodies from PR3-ANCA patients react with plasminogen*

Anti-*cPR3*$^{(138\text{-}169)}$ antibodies were affinity purified from two PR3-ANCA patients using a *cPR3*$^{(138\text{-}169)}$ affinity column. These antibodies were then tested for reactivity to plasminogen by performing a western blot of those fractions that were reactive with the rabbit anti-*cPR3*$^{(138\text{-}169)}$ antibody. The results show that the patients' anti-*cPR3*$^{(138\text{-}169)}$ antibodies react with plasminogen, but not with β_2-glycoprotein-1 like the rabbit antibody, and this reactivity is specific as it can be competed away by pre-incubating the antibody with *cPR3*$^{(138\text{-}169)}$ peptide (Figure 4.1A).

To confirm reactivity, both plasminogen and plasmin were purchased and separated out by SDS-PAGE under reducing and non-reducing conditions. A western blot using patient affinity purified anti-*cPR3*$^{(138\text{-}169)}$ antibody shows that the antibodies only react with non-reduced plasminogen and not with reduced plasminogen or with plasmin (Figure 4.1B).

A target epitope for anti-cPR3$^{(138\text{-}169)}$ *antibodies on plasminogen*

We utilized a mass spectrometry approach to identify an epitope recognized by the patient's anti-*cPR3*$^{(138\text{-}169)}$ antibodies. Antibody was cross-linked to sepharose beads, incubated with plasminogen, and then subjected to a series of proteolytic digestions. Those portions of plasminogen not involved in binding to the antibody are degraded and washed away and the residues on plasminogen in the epitope can be identified by MS/MS analysis. A target epitope was identified for the patient affinity purified anti-*cPR3*$^{(138\text{-}169)}$ antibodies. This epitope, which is part of the catalytic domain of plasminogen, is a surface exposed loop

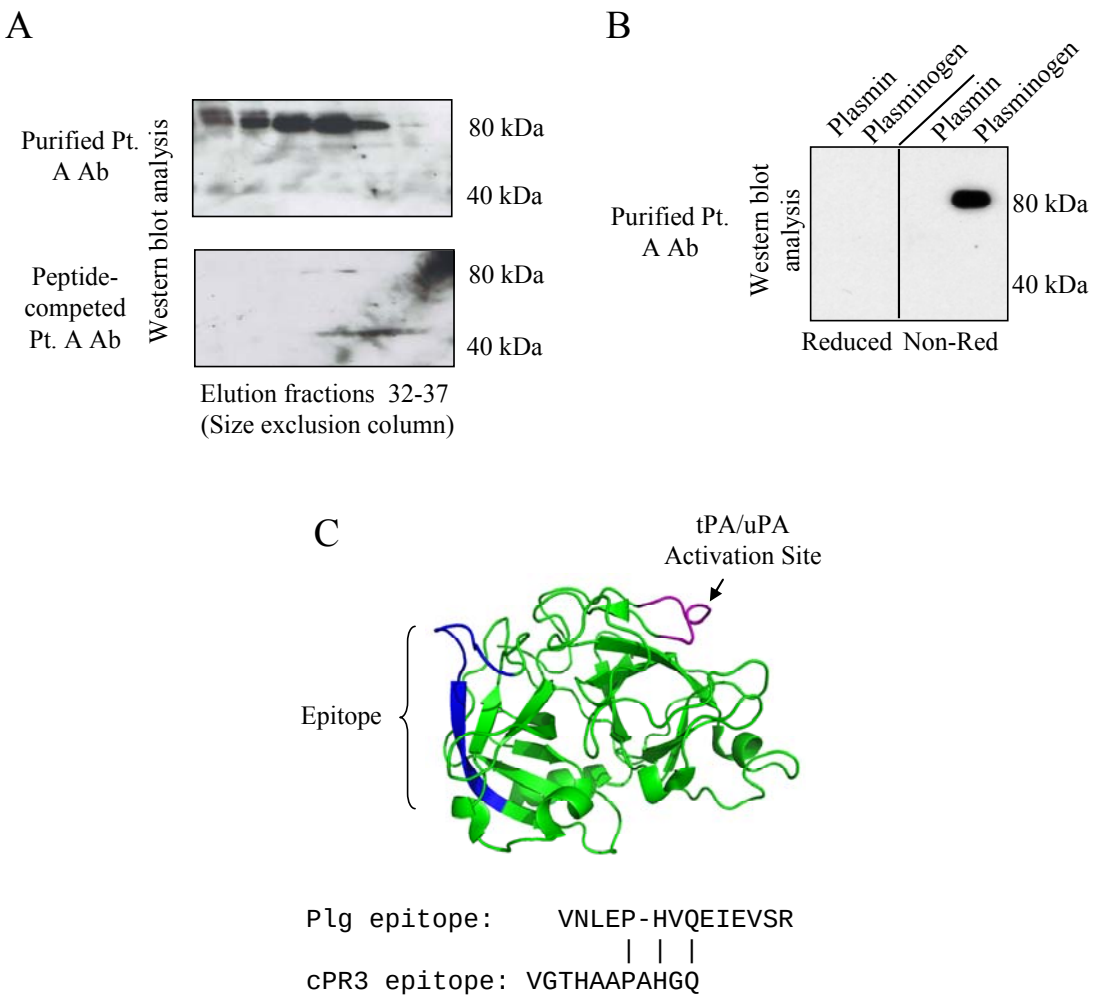

Figure 4.1. Patient IgG, Affinity Purified Using a *cPR3*$^{(138\text{-}169)}$ Peptide Column, Reacts with Plasminogen

(A) Affinity purified patient antibody was used for western blot analysis of protein fractions reactive with the rabbit anti-*cPR3*$^{(138\text{-}169)}$ antibodies. Plasminogen was recognized by the antibody, while β2-glycoprotein-1 was not. When *cPR3*$^{(138\text{-}169)}$ peptide was incubated with patient IgG prior to addition to the nitrocellulose, antibody binding to plasminogen was competed away. (B) Commercially prepared plasminogen and plasmin were separated by SDS-PAGE under both reducing and non-reducing conditions, transferred to nitrocellulose and probed with the patient affinity purified antibody. The patient antibody reacts with non-reduced plasminogen, but not with reduced plasminogen or with plasmin. (C) A cartoon representation of the target epitope of the anti-plasminogen autoantibodies within the catalytic domain of plasminogen (PDB 1DDJ), which is shown in blue. This epitope is shown in relation to the tPA/uPA activation site, which is indicated in purple. The sequences for the plasminogen epitope and the target epitope on cPR3 are shown with the similarity between the two indicated by the underline.

structure residing on the opposite side of the molecule from where plasminogen is cleaved and activated by tPA or uPA (Figure 4.1C). The target sequence (VNLEPHVQEIEVSR) shares little homology with the target sequence of *cPR3*$^{(138\text{-}169)}$ (VGTHAAPAHGQ), however the two sequences do share the common motif P-HXQ. This motif was recognized by two separate patients' affinity purified anti-*cPR3*$^{(138\text{-}169)}$ antibodies as well as an additional three patients total IgG. As a negative control, normal IgG was incubated with plasminogen and evaluated by the same methods. No plasminogen peptides were found to bind to the normal human IgG. Taken together, these results show that anti-*cPR3*$^{(138\text{-}169)}$ and anti-plasminogen antibodies are one in the same.

Assessment of anti-plasminogen autoantibodies on plasminogen function

The epitope of anti-plasminogen antibodies resides in the catalytic domain of plasminogen, which is spatially removed from the tissue-type plasminogen activator (tPA)/urokinase-type plasminogen activator (uPA) cleavage site. Cleavage by tPA or uPA converts plasminogen into plasmin, an active protease capable of fibrinolysis [135]. We tested whether antibody-binding to plasminogen would affect tPA/uPA-induced activation. The first assay examined the effect of anti-plasminogen autoantibodies on plasminogen conversion to plasmin utilizing a chromogenic substrate specific for plasmin. Figure 4.2A shows a sample plot of data generated in this assay. The absorbance at 405 nm increases as the chromogenic substrate is cleaved upon conversion of plasminogen to plasmin in the presence of no antibody, control human IgG or affinity purified patient anti-plasminogen antibody. The patients anti-plasminogen antibody decreased the conversion of plasminogen to plasmin. Two independent experiments were performed for each of two different patient's

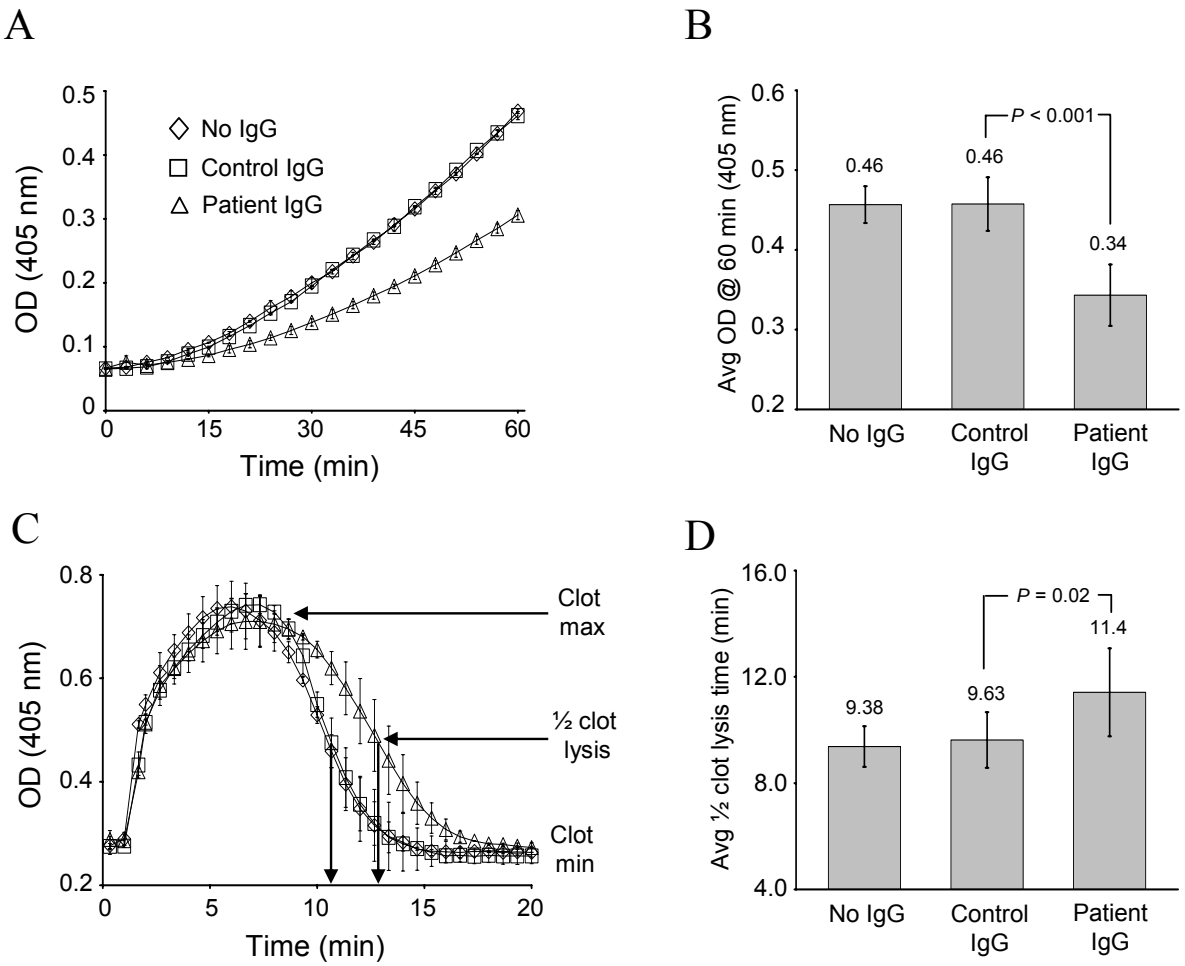

Figure 4.2. Functional Effects of Anti-Plasminogen Autoantibodies.
(A) An *in vitro* assay was performed to determine the rate of plasmin formation in the presence of anti-plasminogen autoantibodies by combining plasminogen, uPA or tPA, and a chromogenic substrate with and without control human IgG or affinity purified autoantibodies. Shown is the average of two replicates for one patient's antibodies in the presence of uPA. (B) The average absorbance after 60 minutes is shown +/- the standard deviation from two independent experiments for Patients A and B with both uPA and tPA. Anti-plasminogen autoantibodies decreased the conversion of plasminogen to plasmin, when compared to HBS buffer or normal human IgG at the same concentration. (C) An *in vitro* clotting assay examined how anti-plasminogen autoantibodies affect fibrin clot formation and/or dissolution. Normal human plasma was combined with HBS buffer alone, normal human IgG or patient anti-plasminogen autoantibodies, in the presence of thrombin and uPA. Clot formation and dissolution were monitored by change in absorbance at 405 nm. Shown is the average of two replicates for one patient's antibodies +/- the standard deviation. (D) The average ½ clot lysis time is shown +/- the standard deviation from two independent experiments for Patients A and B. Anti-plasminogen autoantibodies delayed the fibrinolysis of the clot. Statistical analysis done by Student *t* test.

antibodies using both tPA and uPA. The average OD after 60 minutes was then calculated and plotted in Figure 4.2B. The patients' affinity purified antibodies resulted in a significant decrease in the conversion of plasminogen to plasmin.

The second experiment performed to test the effect of anti-plasminogen autoantibodies on plasminogen activity utilized normal human plasma and examined the effect of clot formation and dissolution. Combining plasma with thrombin and uPA results in a clot forming (as seen by an increase in absorbance at 405 nm) and then dissolving (a decrease in absorbance at 405 nm). Figure 4.2C shows the average results of two independent experiments for one patient's affinity purified anti-plasminogen autoantibodies. The patients' antibodies result in an increase in the time necessary for fibrin clot dissolution. An average of ½ clot lysis time from testing two patient's affinity purified antibodies in two separate experiments showed a significant increase in lysis time when compared to normal human IgG ($P = 0.02$) (Fig. 4.2D). This delay did not involve increased thrombin generation or activation of the thrombin-activatable fibrinolysis inhibitor, as the level of calcium present in the assay was insufficient to cause activation of endogenous clotting factors [136].

Prevalence of anti-plasminogen autoantibodies

The prevalence of anti-plasminogen autoantibodies in a PR3-ANCA patient population was determined by ELISA analysis (Fig. 4.3A). Demographics of study participants are shown in Table 4.1. Anti-plasminogen autoantibodies were higher in the PR3-ANCA patients (16 of 72, 22 %), as compared with 4 of 63 healthy control subjects (6

Table 4.1. Demographics and Clinical Diagnosis of Study Participants

Characteristic	All ANCA-SVV (N = 106)	PR3-ANCA (N = 72)	MPO-ANCA (N = 34)	PR3-ANCA with thrombosis (N = 9)	Idiopathic DVT Control Group (N = 57)
Sex – no. (%)					
Male	56 (52.8)	42 (58.3)	14 (41.2)	6 (66.7)	23 (40.4)
Female	50 (47.2)	30 (41.7)	20 (58.8)	3 (33.3)	34 (59.6)
Race – no. (%)					
White	89 (84.0)	64 (88.9)	25 (73.5)	7 (77.8)	53 (93.0)
Black	10 (9.4)	5 (6.9)	5 (14.7)	2 (22.2)	4 (7.0)
Other	7 (6.6)	3 (4.2)	0	0	0
Age – yr.					
Mean	53.6±19.0	51.2±19.4	58.2±17.8	51.9±19.2	45.1±15.2
Range	13-86	13-86	17-86	15-74	16-86
Clinical Diagnosis – no. (%)					
Wegener's granulomatosis	44 (41.5)	38 (52.8)	6 (17.6)	6 (66.7)	N/A
Microscopic polyangitis	49 (46.2)	29 (40.3)	20 (58.8)	3 (33.3)	
Churg-Strauss Syndrome	4 (3.8)	2 (2.8)	2 (5.9)	0	
Renal limited vasculitis	8 (8.5)	3 (4.2)	6 (17.6)	0	

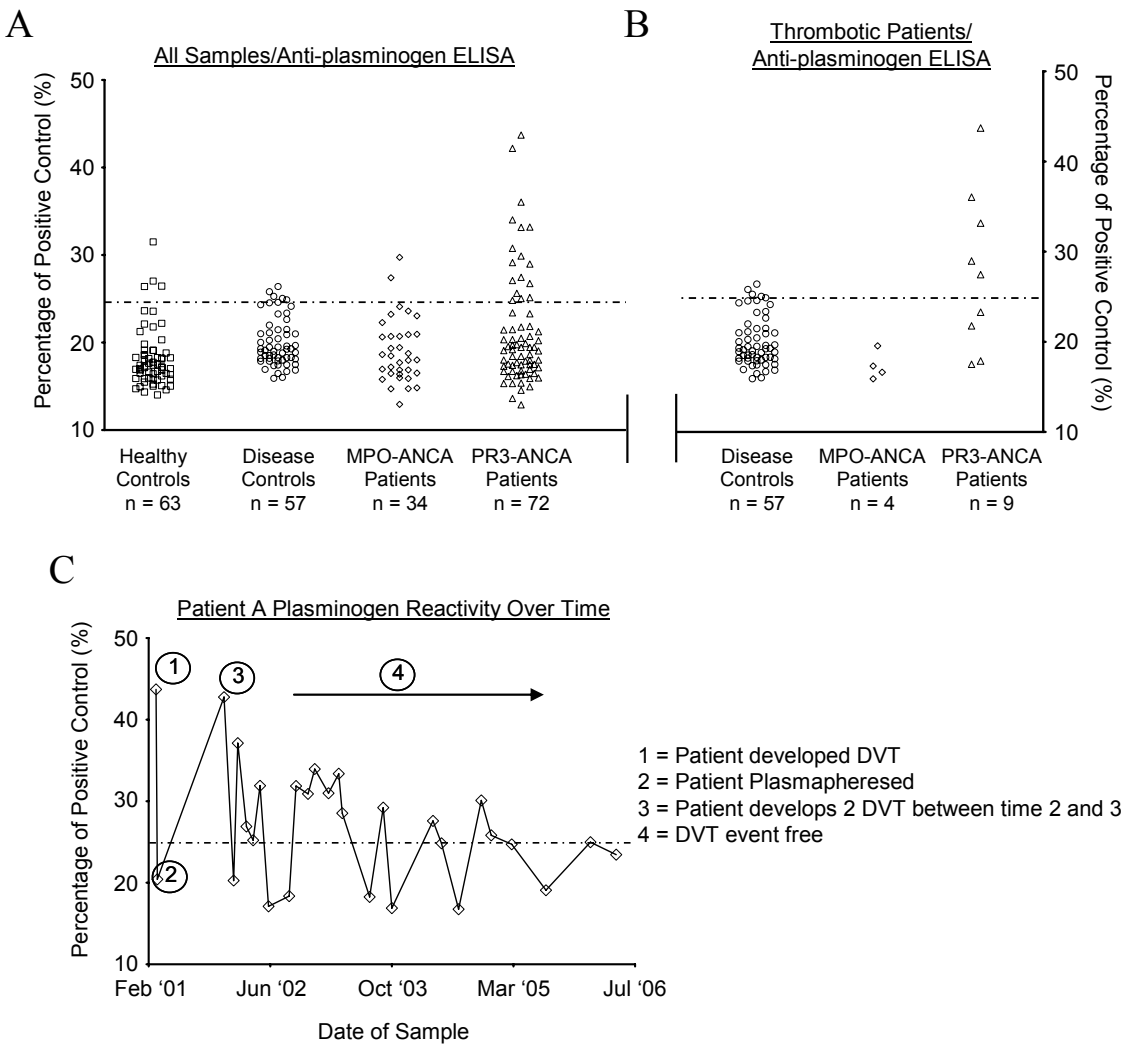

Figure 4.3. Prevalence of Anti-Plasminogen Autoantibodies.
(A) A plasminogen ELISA shows that 16 of 72 (22%) PR3-ANCA patients are positive for anti-plasminogen autoantibodies. This compares with 4 of 63 (6%) healthy control subjects, 5 of 57 (9%) thrombotic patients and 2 of 34 (6%) MPO-ANCA patients. A positive value is defined as two standard deviations above the mean of 63 healthy control subjects (25.6%), as marked by the dashed line. (B) When those patients who had thrombotic events are plotted alone, 5 of 9 (56%) PR3-ANCA patients are positive, compared to 5 of 57 (9%) disease controls and 0 of 4 (0%) MPO-ANCA patients. (C) Multiple samples from Patient A were tested by ELISA to monitor the change in anti-plasminogen autoantibody levels compared to DVT events over time.

%), 2 of 34 MPO-ANCA patients (6 %) and 5 of 57 patients with idiopathic thrombosis (9 %) ($P = 0.001$) (Fig. 4.3A).

Focusing on patients with deep venous thrombosis (DVT), we identified 9 of 72 PR3-ANCA patients with events (6 with Wegener's granulomatosis and 3 with microscopic polyangiitis) (Fig. 4.3B). Of these 9 PR3-ANCA thrombotic patients, 5 were positive for anti-plasminogen autoantibodies (56 %) compared to 0 of 4 MPO-ANCA thrombotic patients (0 %) and 5 of 57 patients with idiopathic thrombosis (9 %) ($P = 0.002$). As assessed by the Birmingham Vasculitis Activity Score (BVAS), the five anti-plasminogen autoantibody positive PR3-ANCA patients had active disease, whereas the four negative sera came from patients in remission with a BVAS score of zero. An extensive workup for thrombophilic defects in all PR3-ANCA patients with a thrombotic event revealed no abnormalities. Importantly, none of the PR3-ANCA thrombotic patients had nephrotic range proteinuria or a history of DVT prior to the onset of the disease. There were 4 patients with MPO-ANCA SVV who similarly developed a thrombosis. No differences were found with respect to risk factors for venous thromboembolism among the PR3-ANCA, MPO-ANCA and the total set of ANCA-SVV patients.

Sera samples from patients collected over a number of years allowed the tracking of autoantibody fluctuations and their association with DVT events. Representative of the data (Figure 4.3C), a patient with a DVT presented with high levels of anti-plasminogen autoantibodies (number 1). The patient received plasmapheresis treatment and the antibody titer dropped (number 2). Over the next nine months the patient experienced two more DVT events and the anti-plasminogen autoantibodies levels were again very high (number 3).

Since that time, the patient has not experienced a DVT and the anti-plasminogen autoantibodies level has trended downward (number 4).

DISCUSSION

Knowing that antibodies raised to *cPR3*$^{(138\text{-}169)}$ peptide reacted with plasminogen from PR3-ANCA patient plasmapheresis fluid (see Chapter III), and the fact that a subset of PR3-ANCA patients had circulating antibodies to this complementary PR3 protein, we sought to determine if PR3-ANCA patients had antibodies that would react with plasminogen. Anti-plasminogen autoantibodies were seen in a subset of PR3-ANCA patients. This autoimmune response was quite restricted in that these autoantibodies reacted with plasminogen but not with plasmin. They react to a motif found on both *cPR3*$^{(138\text{-}169)}$ and plasminogen. These autoantibodies had a functional significance in that they altered fibrinolysis *in vitro* and were associated with a thrombotic propensity in some PR3-ANCA patients.

Plasminogen is a 90 kDa glycoprotein and is the inactive precursor to plasmin. This serine protease is responsible for fibrin clot dissolution [137]. In the presence of fibrin, plasmin is generated by cleavage of the Arg^{561}-Val^{562} bond by tPA [138] or uPA bound to its cellular receptor [139]. Plasminogen is also involved in a number of different pathways including cell migration [140], inflammation [141], and tumorigenesis [142]. These autoantibodies appear to interfere with the ability of plasminogen to dissolve a fibrin clot, but this study did not examine whether or not these autoantibodies alter the activity of plasminogen in any other way. It is interesting that these anti-plasminogen autoantibodies have been shown to affect plasminogen activity *in vitro* despite the fact that their reactivity is highly restricted to plasminogen but not plasmin. Yang et al. identified plasmin-specific autoantibodies in patients with antiphospholipid syndrome that cross-reacted with plasminogen [143]. However, only 1 of 6 patient derived plasmin-specific monoclonal antibodies altered fibrin clot lysis. In solution, these autoantibodies decreased the conversion

of plasminogen to plasmin which may explain the increase in the time necessary to break down a clot in the clotting assay.

Based on these data, we postulated that patients with anti-plasminogen autoantibodies could be more susceptible to clot formation. The fact that 56% of the PR3-ANCA patients studied who were known to have a thrombotic episode also had anti-plasminogen autoantibodies is intriguing, especially considering the serum samples were obtained at times of clinically indicated visits and not at times of clotting episodes. This compares to a study by Simmelink et al. who found no relationship between the presence of anti-plasminogen autoantibodies and development of thrombosis in lupus anticoagulant-positve patients with systemic lupus erythematosus [144]. The disease control patients with idiopathic deep venous thrombosis did not have anti-plasminogen autoantibodies at any higher rate than healthy controls, thus it is unlikely that these autoantibodies are as a consequence of a thrombotic event and are more than likely participating in the cause of them. Myeloperoxidase-ANCA SVV patients, who are very similar with respect to their clinical phenotype, served as a control population. There were only four of these patients who had thrombi and none of them had anti-plasminogen autoantibodies. In this MPO-ANCA SVV patient population there was no significant increase in the overall anti-plasminogen autoantibody propensity when compared to our healthy control population or those with idiopathic thrombotic disorder. In PR3-ANCA patients who had a thrombophilia work-up, there was no laboratory or clinical evidence for increased thrombotic propensity. Our findings may be relevant to a few recent reports that examined venous thrombotic events (VTE) in ANCA vasculitis patients. One study reported 13 of 105 ANCA vasculitis patients

developed a VTE [145]. Ten of these patients were PR3-ANCA positive and three were MPO-ANCA positive, a percentage quite similar to our findings. A second study showed that 29 of 180 patients in a clinical trial of Wegener's granulomatosis developed a venous thrombotic event (VTE) [146]. As a follow up, those investigators examined the 180 patients for presence of anticardiolipin and anti-β_2-glycoprotein-1 antibodies along with several genetic hypercoagulable factors and found no difference between those patients who developed a VTE and those that did not [147].

These autoantibodies do not appear to be long-lasting, thus it is possible that a higher percentage of our patients could have tested positive for anti-plasminogen autoantibodies if they had been purposefully sought at the time of the clotting episode or prior to it. The absence of sera at critical time points is a limitation of our study and a question that needs to be tested in a prospective manner. Conversely, there are patients in our study who had anti-plasminogen autoantibodies but no clinical evidence for a thrombotic event. One explanation, that takes into consideration the *in vitro* data, is that inadequacies in clot dissolution would be of no consequence unless a clot is forming. A second consideration is that aggressive treatment with immunosuppression and plasmapheresis may reduce the titer of anti-plasminogen autoantibodies to levels below a threshold required for phenotypic expression. The autoantibodies may increase the likelihood of a thrombotic event but are not sufficient by themselves to cause such an event, as has been speculated for patients with anti-phospholipid antibodies but no thrombotic episodes.

Our study does not provide any new information as to the inciting cause of PR3 autoantibodies in small vessel vasculitis. This study does demonstrate the presence of a novel and important autoantibody system discovered on the basis of the complementary

protein interaction hypothesis. Interestingly, anti-plasminogen antibodies have also been found in a rat model of human membranous nephropathy known as Heymann nephritis [148], where glomerular lesion results from binding of antibodies to gp330, a receptor of the low-density lipoprotein receptor superfamily that binds to plasminogen [149]. Patients with membranous nephropathy have venous thrombotic episodes, and it would be interesting to determine whether these patients have antibodies to plasminogen as well.

Our studies were designed to explore if PR3-ANCA patients had antibodies to a protein, plasminogen, which had previously been shown to be complementary to the disease autoantigen, PR3. The discovery that autoantibodies to plasminogen are detected in this specific patient population provides additional support for the theory of autoantigen complementarity. The positive correlation between high levels of anti-plasminogen autoantibodies and thrombotic events suggests that the antibodies may have a pathological role in this patient population. Our studies do not illuminate the cause of PR3-ANCA, although there are a number of microbes linked to the onset of ANCA [90, 91] that bear proteins complementary to PR3 [89]. Studying complementary protein interactions provides an elucidation of the perplexing question of why patients with autoimmune diseases have autoimmune responses to structurally different antigens. These studies do provide a novel approach for the discovery of autoantibodies and autoantigens that may have implications in the broadening field of autoimmunity.

CHAPTER V

EPILOGUE

How and why the immune system falters during autoimmune disease is likely to involve a complex series of abnormal situations coming together in precisely the right sequence of events. The primary goal of this research project was to isolate and identify complementary-PR3 proteins that could be implicated in the onset of PR3-ANCA formation according to the theory of autoantigen complementarity. The discovery that PR3-ANCA patients have $CD4^+$ T cells that react with a complementary-PR3 peptide furthers supports the idea that they had an earlier immunological response to a complementary-PR3 protein. The discovery that plasminogen is a complementary-PR3 protein is important and noteworthy, however it can not be considered to be an initiator of PR3-ANCA formation based on its high level of expression in all individuals. Protein F from Pseudomonas was also identified as a complementary-PR3 protein and its potential involvement is intriguing; however the fact that the protein was also identified in a non-ANCA patient makes it less likely that it is a culprit in ANCA development.

Plasminogen does not fit well into the theory of autoantigen complementarity, at least as it is conceived at this point. The sequences of plasminogen and PR3 are not antisense, and plasminogen has only the slightest sequence similarities with $cPR3^{(138\text{-}169)}$. The theory posits that it is a complementary protein, or its microbial mimic, that kicks start an immunological

cascade culminating in the production of autoantibodies. Since plasminogen is such a ubiquitous protein, present in all individuals serum at approximately 250 μg/ml [150], it seems likely that something else is occurring to produce the anti-plasminogen autoantibodies and PR3-ANCA. What is interesting is that the anti-plasminogen autoantibodies and PR3-ANCA form an idiotypic pair. However, this would be true for only those approximately 20% of PR3-ANCA patients that have anti-plasminogen autoantibodies. It is known that different PR3-ANCA patients antibodies recognize different epitopes on PR3. It would not be expected then that all PR3-ANCA would have anti-plasminogen antibodies if those are in fact anti-idiotypic to PR3-ANCA. Thus, it would be interesting to determine if all the PR3-ANCA patients that have antibodies that recognize a certain epitope on PR3 also have anti-plasminogen autoantibodies, and perhaps those patients whose antibodies recognize a different epitope on PR3 also have a different, as of yet undiscovered class of autoantibodies to protein(s) other than plasminogen.

The identification that anti-plasminogen autoantibodies correlate with venous thrombotic events (VTE) in PR3-ANCA patients is especially interesting. Until now, all that was known was that ANCA vasculitis patients had an increased risk for VTE, however it was unclear why this was the case. As we exhibited in Chapter IV, anti-plasminogen autoantibodies taken from two separate patients altered the conversion of plasminogen to plasmin, and also caused an increase in the time necessary for fibrinolysis. The level of calcium was controlled for in these experiments (in order to prevent any activation of endogenous clotting factors), thus it lends support to the autoantibodies involvement. That does not mean it is likely the autoantibodies are the only requirement for VTE, but are most likely one factor that predisposes those individuals who have them to a VTE. A prospective

study will need to be undertaken after a clinical test for anti-plasminogen autoantibodies is developed to study their association with VTE in ANCA vasculitis patients more precisely.

The definition of complementarity as put forth by Root-Bernstein et al. is applicable to the data presented here. Plasminogen and PR3 do not fit the Mekler definition of complementarity, however they do fit the Root-Bernstein definition in that they bind and antibodies to each protein form an idiotypic pair. The real issue then is why does a cPR3 peptide bind to anti-plasminogen antibodies? A possible explanation is that antisense proteins do have complementary shapes to their sense counterpart, and $cPR3^{(138\text{-}169)}$ corresponds to a part of PR3 that binds to plasminogen. Antibodies made to these parts of PR3 and plasminogen then contain the "internal image" of the other. Another way to say this is that $cPR3^{(138\text{-}69)}$ is a mimotope to plasminogen. A mimotope is a peptide that mimics the epitope of a particular protein without sharing sequence homology with that particular protein. Plasminogen and $cPR3^{(138\text{-}169)}$ fit this definition as the two proteins are recognized by the same antibodies and yet they have little sequence homology (outside of the P-HXQ motif). However, that motif is not enough to explain antibody binding as altering the P, H and Q of that motif only resulted in a ~30% decrease in antibody binding. Thus, it appears most likely that a much larger portion of $cPR3^{(138\text{-}169)}$ than the P-HXQ motif is responsible for antibody binding, making $cPR3^{(138\text{-}169)}$ a mimotope of plasminogen.

What are mimotopes and how can they fit in with the results of these studies? Mimotopes are mimics of protein epitopes. They can be utilized for the identification of important amino acid residues in epitope:antibody interactions. Mimotopes were originally described in the study of determinants on foot-and-mouth disease virus. Geysen et al developed a technique whereby a monoclonal antibody directed against foot-and-mouth virus

was used to screen a random peptide library. Those peptides that bound to the antibody were then sequenced and shown to have almost no homology with any portion of the virus capsid proteins [151]. Thus, the researchers had discovered a peptide that bound to antibodies specific for virus capsid proteins with no sequence homology to virus capsid proteins, and they termed these peptides "mimotopes".

The use of mimotopes in disease research was first shown by Balass, et al. while attempting to identify a possible ligand for a monoclonal antibody (mAb 5.5) directed against a conformational epitope on the MG autoantigen AChR [152]. They identified 3 positive clones from a random peptide library with the sequences DLVWLL, DIVWLL and LIEWLL. None of those sequences correspond to any portion of AChR. The monoclonal antibody 5.5 was shown to cause MG in chickens after passive immunization, however administering the peptide DLVWLL specifically blocked onset of MG in mAb 5.5 immunized chickens. In addition to MG, mimotopes have been discovered that bind antibodies raised to the autoantigens of ITP [153], primary biliary cirrhosis [154] and SLE [155]. Mimotopes have also been used for immunization studies, as peptides from randomly generated libraries have been shown to induce a protective immune response to measles virus [156] and to pneumococci [157]. None of the peptides used in the immunizations bore any sequence homology to the known epitope determinants recognized by the antibody used to discover the peptide.

Now that a mimotope of plasminogen has been identified and used for the discovery of anti-plasminogen autoantibodies, there are at least two potential applications for mimotopes in ANCA research. The first is to determine if screening of a mimotope library can be used to epitope map PR3-ANCA and MPO-ANCA, as all efforts to do so up to this

point have been met with limited success. Unfortunately, since both sets of antibodies appear to bind conformational epitopes, identification of peptides that contain sequences of PR3 or MPO is unlikely. However, a more promising avenue of research may be with the use of mimotopes for the treatment of ANCA disease. Knowing that mimotopes can bind to antibodies that recognize a certain protein, the question then becomes whether they can be utilized for the *prevention* of antibody binding to protein, i.e. can they block autoantibodies from binding to autoantigen in autoimmune disease? A potential set of experiments would be to use patient sera, or purified PR3-ANCA, to screen random peptide libraries for the discovery of mimotopes that would bind to ANCA and prevent binding to PR3, thereby inhibiting their pathogenic capabilities. Alternatively, since there is an animal model of MPO-ANCA mediated disease it would be possible to identify mimotopes that bind to MPO-ANCA and determine if they can be successfully administered in mice to prevent disease caused by immunization with MPO-ANCA. While $cPR3^{(138\text{-}169)}$ would not make a good mimotope candidate due to the fact that it is antisense to PR3 and invokes the idiotypic network (i.e. anti-plasminogen antibodies), it has still opened up the possibility for an entirely new avenue of research into treatment options for this autoimmune disease.

The theory of autoantigen complementarity remains a viable model for the induction of autoimmune disease, however the identification of a complementary protein that can be utilized in causation of disease in an animal model remains elusive. While a proximal antigen was not discovered, these studies have shown that protein complementarity can be utilized for the identification of novel autoantibodies and autoantigens, and the search for a disease-causing complementary protein should continue.

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