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Collin, Mattias

2001

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Citation for published version (APA):

Collin, M. (2001). *Streptococcus pyogenes secreted enzymes interacting with the human host*. [Doctoral Thesis (compilation), Infection Medicine (BMC)]. BMC Biomedical Centre, Lund University.

Total number of authors:

1

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Streptococcus pyogenes secreted enzymes
interacting with the human host

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Lund University
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***Streptococcus pyogenes* secreted enzymes interacting with the human host**

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Akademisk avhandling

som med vederbörligt tillstånd från Medicinska fakulteten vid Lunds Universitet för avläggande av doktorexamen i medicinsk vetenskap kommer att offentlig försvaras i Fernströmsalen, Biomedicinskt centrum, Sölvegatan 19, Lund, fredagen den 9:e november 2001, klockan 13.15.

Fakultetsopponent

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FACULTY OF MEDICINE
Lund University

Organization LUND UNIVERSITY Department of Cell and Molecular Biology Section for Molecular Pathogenesis	Document name DOCTORAL DISSERTATION	
	Date of issue 9 November 2001	
	Sponsoring organization	
Author(s) Mattias Collin		
Title and subtitle <i>Streptococcus pyogenes</i> secreted enzymes interacting with the human host		
Abstract <p><i>Streptococcus pyogenes</i> is one of the most common bacteria infecting humans. One of the factors contributing to the disease-causing properties is the secreted streptococcal cysteine proteinase, SpeB, which degrades several host proteins in connective tissue and circulation. SpeB activity depends on the transformation from an inactive precursor, zymogen, into a mature proteinase. We show that the important cell wall-anchored M protein is involved in the unfolding and maturation of SpeB. An engineered strain lacking M protein secreted the SpeB zymogen in a conformational state that did not allow the maturation to proceed normally. Furthermore, we identified genes that were regulated by environmental changes using RNA fingerprinting. One of the identified glucose-induced genes encodes a phosphotransferase system that could be involved in the signaling leading to down-regulation of virulence genes in response to the nutritional status. Moreover, a novel secreted endoglycosidase, EndoS, has been identified. Purified EndoS removes the glycan from native human immunoglobulin G (IgG). This glycan is known to be important for IgG effector functions such as complement activation and binding to Fc receptors on phagocytic cells. Furthermore, it was discovered that SpeB cleaves the heavy chain of IgG resulting in distinct fragments separating the antigen-binding Fab portions from the effector-triggering Fc portion. SpeB also degrades the heavy chains of IgA, IgM, IgD and IgE. Finally, it was shown that both these enzymatic activities on human IgG have functional consequences with significantly impaired antibody-mediated killing of <i>S. pyogenes</i> in an <i>ex vivo</i> model.</p>		
Key words: <i>Streptococcus pyogenes</i> , cysteine proteinase, RNA fingerprinting, endoglycosidase, immunoglobulins, glycosylation, autoimmunity		
Classification system and/or index termes (if any):		
Supplementary bibliographical information:	Language English	
ISSN and key title:	ISBN 91-628-4966-2	
Recipient's notes	Number of pages 138	Price
	Security classification	

Distribution by (name and address) Mattias Collin, CMB, BMC B14, Tornavägen 10, 221 84 Lund
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Date 21 September 2001

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**Department of Cell and Molecular Biology
Section for Molecular Pathogenesis
Biomedical Center**



FACULTY OF MEDICINE

Lund University

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Streptococcus pyogenes secreted enzymes

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Printed in Sweden
KFS AB, Lund 2001
ISBN 91-628-4966-2

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To Eva

A small bacterium weighs as little as 0.0000000001 gram.

A blue whale weighs about 100,000,000 grams.

Yet a bacterium can kill a whale...

Microbes, not macrobes, rule the world.

Bernard Dixon

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LIST OF ABBREVIATIONS

α_2 M	α_2 -macroglobulin
ADCC	antibody-dependent cell-mediated cytotoxicity
APSGN	acute post-streptococcal glomerulonephritis
ARF	acute rheumatic fever
Bp	base pair
C (number)	complement factor (number)
C4BP	C4b-binding protein
CcpA	catabolite control protein
cDNA	complementary DNA
ECM	extracellular matrix
EndoS	endoglycosidase of streptococci
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GlcNAc	<i>N</i> -acetylglucosamine
GRAB	protein G-related α_2 -macroglobulin-binding protein
HDL	high-density lipoprotein
HPr	Histidine protein
IBD	inflammatory bowel disease
Ig	immunoglobulin
MAC	membrane attack complex
MF	mitogenic factor
NADase	NAD ⁺ -glycohydrolase
N-linked	asparagine-linked
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PTS	phosphotransferase system
RA	rheumatoid arthritis
RAP	RNA fingerprinting by arbitrarily primed RT-PCR
RF	rheumatoid factor
SAGP	streptococcal acid glycoprotein
Scl (A, B)	streptococcal collagen-like protein (A, B)
SCPA	C5a peptidase
SDH	streptococcal surface dehydrogenase
SEN	streptococcal surface enolase
SGSP	Streptococcal Genome Sequencing Project
Sic	streptococcal inhibitor of complement
SLE	systemic lupus erythematosus
SLO	streptolysin O
SLS	streptolysin S
SOD	superoxide dismutase
SOF	serum opacity factor
Spe (A, B, C)	streptococcal pyrogenic exotoxin (A, B, C)
TSS	toxic shock syndrome

LIST OF PUBLICATIONS

This thesis is based on the following publications referred to in the text by their Roman numerals (I-V):

- I. Collin, M. and Olsén, A. (2000) Generation of a mature streptococcal cysteine proteinase is dependent on cell wall-anchored M1 protein. *Molecular Microbiology*. **36**, 1306-1318.
- II. Collin, M. and Olsén, A. (2001) Identification of conditionally expressed genes in *Streptococcus pyogenes* using RNA fingerprinting. *FEMS Microbiology Letters*. **196**, 123-127.
- III. Collin, M. and Olsén, A. (2001) EndoS, a novel secreted protein from *Streptococcus pyogenes* with endoglycosidase activity on human IgG. *The EMBO Journal*. **20**, 3046-3055.
- IV. Collin, M. and Olsén, A. (2001) Effect of SpeB and EndoS from *Streptococcus pyogenes* on human immunoglobulins. *Infection and Immunity*. **69**, In press.
- V. Collin, M. and Olsén, A. (2001) SpeB and EndoS mediate *Streptococcus pyogenes* survival in human blood. Submitted to *EMBO Reports*.

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INTRODUCTION

Humans are constantly colonized by microorganisms on the outer linings of the body, such as on the skin and on mucosal surfaces in the upper airways, gastrointestinal tract, and lower urogenital tract. In contrast, the interior of the human body is kept free from microorganisms by physical barriers and the components of the innate and adaptive immune responses. Most of the colonizing microorganisms are harmless or even beneficial for the host and do not cause disease or penetrate these barriers. However, some microorganisms that cause local disease in the mucosa or skin can under certain circumstances disseminate into deeper tissue and spread via the blood stream or lymphatic system.

The Gram-positive bacterium *Streptococcus pyogenes*, investigated in this thesis, has the ability to cause disease, both relatively mild local disease as well as life-threatening invasive or systemic disease. Therefore, the main subject for the thesis was to study the regulation and effects of secreted enzymes from *S. pyogenes* in relation to the human host. The aim was to increase the understanding of the underlying molecular events controlling the relationship between *S. pyogenes* and the human host and the development of disease. This could also be described as studies of the molecular pathogenesis of *S. pyogenes*.

This thesis gives an introduction to *S. pyogenes* diseases and pathogenicity, but also an overview of the extracellular enzymes and their role in virulence. Furthermore, there is an introduction to bacterial phosphotransferase systems involved in carbohydrate transport and intracellular signaling, as well as an overview of the structure and function of immunoglobulins. In the present investigation section, there is a summary of the original findings presented in the publications, on which this thesis is based. These include; 1) the study of the maturation of the streptococcal cysteine proteinase, SpeB; 2) the utilization of RNA fingerprinting to identify conditionally expressed genes; 3) the identification of EndoS, a novel endoglycosidase; 4) the characterization of SpeB and EndoS activities on other immunoglobulins; and finally 5) an investigation of how *S. pyogenes* evades the adaptive immune response.

Streptococcus pyogenes

The Gram-positive bacterium streptococcus was discovered already in 1879 by Louis Pasteur in a sample from a patient who died from puerperal sepsis, and the generic name *Streptococcus* was proposed by Rosenbach in 1884. The species

now referred to as *S. pyogenes* was originally denoted *S. haemolyticus* due the characteristic clear zone of hemolysis around the bacteria when grown on blood-agar plates. The bacteria are also referred to as group A streptococci, based on the immunological properties of the so-called group carbohydrate in the cell wall polysaccharide. The group A carbohydrate is composed of *N*-acetyl- β -D-glucosamine linked to a polymeric rhamnose backbone, distinguishing this group from the other streptococci, such as group B, C, F and G. The bacteria grow as indicated by their name, as a chain of beads. Around the cytoplasmic membrane is a thick cell wall composed of repeated peptidoglycan units that provide stability and serves as anchoring points for the cell wall-attached proteins. Furthermore, some strains secrete hyaluronic acid, which forms a capsule that gives bacterial colonies a mucoid (glossy) appearance when grown on solid media.

S. pyogenes may be further subdivided into serotypes based on the composition of surface proteins. The most widely used typing is based on the antigenic properties of the cell wall-anchored M protein (135,136). In addition to classical method using type-specific antiserum, molecular M-typing techniques have been developed based on sequence variability in the part of the *emm* gene that encodes the NH₂-terminal tip of the M protein (12). Furthermore, the T surface antigen has been proven useful to distinguish between strains particularly, when M-typing is not possible, since there is a correlation between T- and M-type (11). Typing of strains could also be performed assaying the serum opacity factor, since the serological response against this protein also correlates with the M-type (264).

The complete genome from a *S. pyogenes* strain of M1 serotype has now been sequenced and annotated. The genome consists of 1,852,442 bp and contains 1,752 predicted protein-encoding genes (66). The starting point of this sequencing project was a traditional chromosomal mapping of the prototype M1 strain SF370 (244). Furthermore, the replication machinery of this strain as well as other *S. pyogenes* strains have been characterized (25,245). During the sequencing process of the *S. pyogenes* genome in the Streptococcal Genome Sequencing Project (SGSP), the database has been publicly available to the research community via the Internet (221). This has enhanced the possibilities for identification and characterization of individual genes and proteins. Furthermore, a more global approach in the identification of gene- or protein families has been made possible. For instance, a broad identification of immunogenic culture supernatant proteins by two-dimensional protein electrophoresis and N-terminal sequencing was greatly facilitated by the use of SGSP database (139).

***Streptococcus pyogenes* secreted enzymes**

Furthermore, an improved sequence pattern was used for *in silico* identification of cell wall-anchored proteins in *S. pyogenes* as well as other Gram-positive bacteria (106). In addition, global differential gene expression in response to growth temperature was investigated using DNA microarrays constructed from SGSP sequence data (236).

The SGSP database was also used to identify conditionally expressed genes of *S. pyogenes* by RNA-fingerprinting (**Paper II**). Also, the identification of a novel secreted endoglycosidase was greatly facilitated by using information from the database in combination with traditional protein sequencing as well as molecular biology and biochemical characterization methods (**Paper III**).

Diseases caused by *Streptococcus pyogenes*

Acute infections

Streptococcus pyogenes is one of the most common human pathogens. *S. pyogenes* is the most prevalent bacterial causative agent of pharyngitis, and accounts for 15 to 30 percent of all pharyngitis cases in children, and 5 to 10 percent in adults (19). Besides infections in the upper respiratory tract, it also infects skin and soft tissue, especially among people living in warm and humid climates. Most of these infections, as impetigo, erysipelas, and cellulitis, are localized to the skin (20). However, in a significant proportion of these infections bacteria disseminate into deeper tissue. This can lead to necrotizing fasciitis, an infection of the subcutaneous tissue with substantial destruction of fascia and fat. Further dissemination of the bacteria can ultimately lead to sepsis and a toxic shock syndrome (TSS), and the incidence of this type of serious infections has increased lately (187,238). TSS is a serious condition with hypotension and multi-organ failure, where 30-60% of the patients die within 72-96 hours (239). Recent epidemiological studies have shown that a few M serotypes, particularly M1 and M3, are over-represented among the serious infections (43,112).

Post-infectious conditions

In addition to the acute *S. pyogenes* infections, there are a number of aseptic sequelae affecting different organ systems. These include acute post-streptococcal glomerulonephritis (APSGN), which can lead to renal failure, and acute rheumatic

fever (ARF) with joint inflammation, carditis, as well as symptoms from the central nervous system and skin manifestations.

One proposed theory of the pathogenesis of ARF is that the host develops an autoimmune response against heart tissue triggered by bacterial proteins mimicking host molecules, i.e. M protein (1,172,271). ARF is still one of the major causes of cardiovascular disease in developing countries responsible for 25-45% of the cases (189). ARF develops after throat infections but not after skin infections, most likely due to the richness of lymphoid tissue in the pharyngeal region. Certain serotypes are considered more rheumatogenic than others are, and strains of M3 and M18 serotype are commonly associated with ARF (112).

APSGN can follow both pharyngeal and skin infections with *S. pyogenes* after a latency period of 1 to 4 weeks. The disease presents with edema, hypertension, hematuria, urinary sediment abnormalities, and decreased serum complement levels. Several pathogenic mechanisms for APSGN have been proposed, including immune complex deposition, cross-reacting antibodies, alteration of glomerular tissue by secreted streptococcal products, and complement activation by streptococcal components deposited in the kidney [(48) and references therein]. It has been shown that circulating immune complexes in APSGN patients contain streptococcal antigens (73), and that IgG deposited in the kidney leads to glomerular changes due to complement activation (28). Furthermore, antiglomerular antibodies eluted from the kidney reacts with the M12 protein (144). This suggests that molecular mimicry between streptococcal antigens and kidney is important for the development of APSGN. Moreover, the secreted plasminogen activator streptokinase is associated with APSGN since strains of M49 serotype deleted in the *ska* gene, encoding streptokinase, did not cause glomerulonephritis in a mouse model (185). Furthermore, the streptococcal cysteine proteinase, SpeB, has been suggested to play a role in the development of APSGN (47). Patients with this condition have elevated antibody levels against SpeB, and the protein can be detected in glomerulonephritis biopsies (46).

Pathogenicity and virulence of *Streptococcus pyogenes*

The concept of pathogenicity, the ability to cause disease, is often debated and definitions tend to vary considerably (32). A generally used definition of a pathogen is that it causes disease in a host. This microbe-centered view of pathogenicity does not take into account that some “pathogens” does not cause disease in all

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hosts it colonizes. This is also true for *S. pyogenes* that in some individuals colonizes the upper respiratory tract without causing any disease (191). Furthermore, *S. pyogenes* does not cause disease in immune hosts that have generated specific antibodies to a particular serotype during previous infections (48).

Another central term in microbial pathogenesis is virulence. This term is currently used to define the relative capacity of a microbe to cause disease and that this is a feature that distinguishes pathogens from non-pathogens. Individual microbial components, virulence factors, have often been defined as factors that when deleted diminish virulence, but not viability of the organisms (33). Most studies of *S. pyogenes* virulence have used this definition, even though not always clearly stated. In this thesis, this definition will be used in the review of known virulence factors, as well as potential new ones, unless stated otherwise.

A number of individual bacterial components have been described as, or suggested to be, virulence factors of *S. pyogenes*. These include surface structures and secreted molecules. This thesis mainly reviews and discusses *S. pyogenes* secreted enzymes and their role in pathogenicity, but begins with a brief overview of virulence factors of all types.

Surface virulence factors

The cell wall-anchored M protein is known to contribute to bacterial survival in human blood, and has therefore been attributed antiphagocytic properties (136). M protein, together with the M-like and M-related proteins are known to specifically interact with a number of host plasma proteins, a feature that may contribute to virulence. These include interactions with components of the coagulation and contact systems such as fibrinogen, H-kininogen, and plasminogen (13,17,117); binding of regulating factors in the complement system such as factor H, factor H-like protein 1, and C4b-binding protein (C4BP) (92,129,252); binding of immunoglobulins G and A (3,143,219); and binding of other host molecules such as the keratinocyte receptor CD46, serum albumin and fibronectin (4,71,188) [for a recent review on surface proteins of Gram-positive bacteria, see (180)]. Several studies have shown that M protein contributes to the virulence of *S. pyogenes* both in cellular adhesion and invasion *in vitro*, and in animal models of infection (7,50,57,90,258). The interactions between *S. pyogenes* and immunoglobulins will be further discussed in the section **“Bacterial interactions with immunoglobulins”**.

Several other surface molecules have been proposed to be involved in the virulence of *S. pyogenes*. These include the cell wall-anchored lipoproteinase serum opacity factor (SOF) that binds fibronectin (44,214,227), the complement inhibiting C5a peptidase (262), protein F that binds fibronectin and promotes cellular adhesion and invasion (81,103,248), the α_2 -macroglobulin-binding protein GRAB (217) and the two collagen-like surface proteins SclA and SclB (155,156,215,216,263). Furthermore, there are the streptococcal surface dehydrogenase (SDH) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity, and the streptococcal surface α -enolase (SEN) that binds plasmin and fibronectin (148,192,195), as well as the cell wall component lipoteichoic acid that contributes to adherence, exerts cytotoxic effects on cells and thereby inhibits bacterial entry into cells (45,231). The surface proteins with enzymatic activities will be further discussed in the chapter “**EXTRACELLULAR ENZYMES**”.

Secreted virulence factors

A number of secreted products have been implicated in *S. pyogenes* virulence. These include exotoxins, superantigens, cytolytins, and several types of enzymes. For instance, the pyrogenic exotoxins A and C (SpeA and SpeC), which have superantigenic activity inducing T-cell proliferation without antigen presentation. These have been suggested to participate in the development of toxic shock due to a massive inappropriate immune response (130). The DNase mitogenic factor (MF), the streptococcal superantigen SSA, as well as the streptococcal cysteine proteinase also have superantigenic activity, and in the case of SpeB, this activity is independent of enzymatic activity (62). The cytolytic toxin streptolysin S (SLS) is responsible for the hemolytic property of *S. pyogenes* and has been shown to be a virulence factor in a mouse infection model (18,184). The pore-forming, thiol-activated cytolytic streptolysin O (SLO) co-operates with other virulence factors and thereby augments tissue damage and inflammatory responses (223,232). In addition, mice infected with SLO-deficient mutant bacteria showed decreased mortality when compared with wild type bacteria (142). Furthermore, SLO is a functional equivalent of type III secretion in Gram-negative bacteria. SLO aids other secreted products such as the NAD⁺-glycohydrolase (NADase) to penetrate the membrane of host cells and thereby induce cytotoxicity (160). In an epidemiological study, NADase was expressed by *S. pyogenes* strains associated with an outburst of TSS (240). Another secreted possible virulence factor is the streptococcal inhibitor of complement (Sic) that inhibits formation of the

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complement membrane attack complex (MAC), by preventing uptake of complement factors C567 onto cell membranes (5,65). Inactivation of the *sic* gene in a strain of M1 serotype resulted in decreased mucosal colonization in mice (153). Another secreted virulence factor is hyaluronan that forms a capsule around the bacterium, protecting it from phagocytosis and cellular uptake (229,261). Furthermore, both the plasminogen activator streptokinase and the streptococcal hyaluronidase have been implicated in the penetration of the extracellular matrix (ECM) (102,133). Another secreted factor that has been associated with *S. pyogenes* nephritogenic properties is the neuraminidase that releases sialic acid from glycoproteins (177). The secreted factors with enzymatic activities will be further discussed in the following chapter.

EXTRACELLULAR ENZYMES

S. pyogenes expresses a number of different enzymes that could be of importance for the host-parasite interaction. When studying these enzymes, it could be practical to divide them into groups or families that share common properties. This division of enzymes could be based on their location, enzyme type, enzyme structure, or on properties and functions of their substrates or ligands. This grouping of enzymes will be partly overlapping, but may provide a starting point for a discussion on these enzymes from different points of view. A summary of the described enzymatic activities of *S. pyogenes* is presented in Table I on page 18.

Location of enzymes

The location of an enzyme is important when trying to understand the relevance of an isolated enzymatic activity with respect to its interaction with a host. Large sets of intracellular enzymes are crucial for the basic metabolic and genetic machinery of any bacterial cell, but such enzymes will not be discussed here. This study is focused on extracellular enzymes and their involvement in the host-parasite interaction. Therefore, mainly secreted and surface associated enzymes of *S. pyogenes* will be discussed from hereon. It should be noted though, that some of the enzymes that have been shown to be extracellular and play a role in pathogenesis, are enzymes normally regarded as intracellular enzymes.

One of the most studied secreted enzymes is the streptococcal cysteine proteinase (61) also referred to as SpeB (76). Other secreted enzymes are the

plasminogen-activating streptokinase (93), as well as the superantigen mitogenic factor (MF) (101) and a NAD⁺-glycohydrolase (2). Evidence has also been presented that *S. pyogenes* expresses an extracellular manganese-dependent superoxide dismutase (SOD) that detoxifies superoxide radicals (77). Furthermore, *S. pyogenes* secretes EndoS, an endo- β -*N*-acetylglucosaminidase that hydrolyzes the N-linked glycan on human IgG (**Paper III**).

Surface associated enzymes can be divided into three main groups. 1) Proteins that are covalently attached by the enzyme sortase (169) to the peptidoglycan cell wall via a carboxy-terminal LPXTG-motif (68), 2) lipoproteins anchored in the plasma membrane via a covalently attached lipid moiety (108), 3) proteins associated by other means to the bacterial surface, probably based on charge and/or hydrophobic interactions. To the LPXTG-anchored group could be added the complement-inhibiting C5a peptidase (262), the lipoproteinase serum opacity factor (SOF) (227), as well as a recently identified serine protease with unknown function (106,139). In the lipoprotein group, no *S. pyogenes* extracellular enzyme has been described to date, but surface lipoproteins often constitute the extracellular component of ABC-transporters with membrane-associated ATPase activity (105,206). To the last group of enzymes, with unknown anchoring mechanisms, SEN and SDH belong (192,195), even though data has been presented that the cell wall-anchored M proteins could be responsible for the surface association of SDH (49). Furthermore, the DegP protease has been suggested to be surface associated (113). Recent data suggest that SpeB could be placed also in this group, since it is believed to be partly surface associated by a yet unknown mechanism (100). Furthermore, the cell wall-anchoring enzyme sortase has its activity closely associated with the cell wall and is most likely a transmembrane protein (169).

Types of enzymes

Enzymes produced by *S. pyogenes* could be grouped by similarities in structure as well as substrate preferences. One distinct group is constituted by the polypeptide-hydrolyzing proteases. SpeB and the C5a peptidase (42) are both proteases that can be further subdivided into cysteine proteinases and serine proteinases, respectively based on the amino acid composition of their active sites. In addition, both the highly conserved DegP protease that is involved in degradation of misfolded proteins (113), as well as the secreted streptokinase also belong to the

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serine proteinases, but streptokinase is in itself enzymatically inactive. However, activation of plasminogen by streptokinase leads to unregulated plasmin activity at the bacterial surface (149). Furthermore, the serum opacity factor, a lipoproteinase that hydrolyzes apolipoproteins in high-density lipoproteins in serum, has been suggested to be an aspartic proteinase (226). Another enzyme that is crucial for the cell wall-anchoring of surface proteins is the sortase. This specific transpeptidase cleaves proteins with the LPXTG-motif and anchors them in the peptidoglycan cell wall (68,179). The enzyme was first characterized in *Staphylococcus aureus* by Schneewind and colleagues that in addition identified a nearly identical sortase gene in *S. pyogenes* (169).

Another group of enzymes that hydrolyze carbohydrates include the hyaluronan-degrading hyaluronidase (97) and the sialic acid-releasing neuraminidase (83). Furthermore, a recently identified putative pullulanase (106), the extracellular glycolytic enzyme SDH that also has ADP-ribosylating activity (193), and the α -enolase SEN (195) belong to this group of enzymes. In addition, the secreted endoglycosidase EndoS hydrolyzes asparagine-linked glycans on IgG (**Paper III**).

Nucleic acids are substrates for a group of enzymes that includes MF, which is a heat stable nuclease (101), and possibly a newly identified cell wall-anchored putative nucleotidase (106). In addition *S. pyogenes* secretes a NADase that hydrolyzes NAD⁺ into adenosin-diphosphoribose and nicotinamide, but also synthesizes the signaling molecule cyclic ADP-ribose (121). NADase is aided by SLO to translocate into host cells where it induces cytotoxicity (160).

An enzyme belonging to another family is the streptococcal acid glycoprotein (SAGP), originally described as an antitumour protein (115). SAGP has arginine deiminase activity that inhibits T-lymphocyte proliferation (53). In addition, this protein is important for bacterial survival at low pH, possibly contributing to intracellular survival (52).

Properties and functions of substrates

Some clues to the substrate preference of *S. pyogenes* enzymes are of course given by the type it belongs to; proteases degrades or cleaves polypeptides, glycolytic enzymes digest carbohydrates, and nucleic acids are hydrolyzed by enzymes belonging to the nuclease or nucleotidase families. However, in order to further understand the relevance of these enzymatic activities in the host-parasite

interaction, the location and functional properties of the substrate molecules needs to be considered. For instance, is the substrate readily accessible in the closest surroundings of the bacteria during the different stages of infection? Furthermore, is the substrate a structural component of for instance the extracellular matrix, a plasma protein, an immunologically important molecule, a component of a homeostatic system such as an inhibitor or activator of important pathways, or finally a surface-attached or secreted molecule from the bacterium itself? Moreover, several interactions between *S. pyogenes* enzymes and host molecules do not involve hydrolysis but rather work as targeting or adhesive interactions. Some of these aspects will be discussed in the following chapters where some of the introduced enzymes are described in more detail.

Streptococcus pyogenes secreted enzymes

Table I. Enzymatic activities expressed by Streptococcus pyogenes

Enzyme	Location	Type of enzyme	Activities	References
C5a peptidase	LPXTG-anchored	Serine proteinase	Cleaves the complement factor C5a	(262)
DegP	Surface associated	Serine proteinase	Temperature and oxidative resistance	(113)
EndoS	Secreted	Endo- β -N-acetylglucosaminidase	Hydrolyzes the N-linked glycan on IgG	Paper III
Hyaluronidase	Secreted	Hyaluronate lyase	Degrades hyaluronic acid	(97, 133, 170)
Mitogenic factor	Secreted	Nuclease	Superantigen and heat stable nuclease activity	(78, 101)
NADase	Extracellular?	NAD ⁺ - glycohydrolase,	Alters neutrophil migration	(121, 160, 240)
		ADP-ribosyltransferase	Translocates into cells mediated by SLO	
Neuraminidase	Secreted	Sialidase	Releases sialic acid from glycoproteins	(51, 177)
			Associated with nephritogenic properties	
Nucleotidase	LPXTG-anchored	Putative nucleotidase	Unknown	(106)
Pullulanase	LPXTG-anchored	Putative pullulanase	Unknown	(106)
SAGP	Extracellular?	Arginine deiminase	Antitumour protein	(52, 53, 115)
			Inhibition of T-cell proliferation	
SOF	LPXTG-anchored	Aspartic apolipoproteinase	Cleavage of apoproteins in HDL, fibronectin binding	(44, 226, 227)
SOD	Extracellular	Superoxide dismutase	Detoxification of oxygen radicals	(77)
Sortase	Cell wall associated	Transpeptidase	Anchoring of LPXTG-proteins to the cell wall	(66, 169)
SpeB	Secreted	Cysteine proteinase	See Table II	See Table II
SDH	Surface associated	Glyceraldehyde-3-phosphate dehydrogenase, ADP-ribosylation	Binding of fibronectin, actin, myosin, and plasmin	(192-194)
Streptokinase	Secreted	Inactive serine proteinase	Signal transduction into host cells	(14, 93)
			Plasminogen activation at the bacterial surface	
			Associated with nephritogenic properties	
Subtilisin-like protein	LPXTG-anchored	Putative serine proteinase	Unknown	(106, 139)
SEN	Surface associated	α -enolase	Plasmin (ogen) binding	(69, 195)

The streptococcal cysteine proteinase, SpeB

The streptococcal cysteine proteinase is the most extensively studied secreted protein from *S. pyogenes*. Its enzymatic activity was first discovered by Stuart Elliott already in the 1940's (61). In the literature, the enzyme is also referred to as the streptococcal pyrogenic exotoxin B (SpeB) since it was discovered that these two secreted proteins are identical (76,82). The gene encoding SpeB is 1,194 bp and has been shown to be present and highly conserved in essentially all *S. pyogenes* isolates (270). The protein is 398 amino acids with a 28 amino acids signal peptide (Ss) that after removal leaves a 40 kDa zymogen form of the enzyme. To generate a mature 28 kDa mature proteinase, the propeptide needs to be cleaved off. The active site consists of a single cysteine, Cys-192, and a histidine, His-340 (145,147,247), and the mature proteinase is dependent on reducing conditions in order to be enzymatically active (147) (for a schematic map of SpeB, see figure 1). The crystal structure of the zymogen was recently solved revealing a fold similar to another cysteine proteinase, papain, despite negligible sequence identity. Furthermore, the active site in SpeB is a catalytic dyad Cys-His as compared to the Cys-His-Asn catalytic triad usually found in other cysteine proteinases (114).

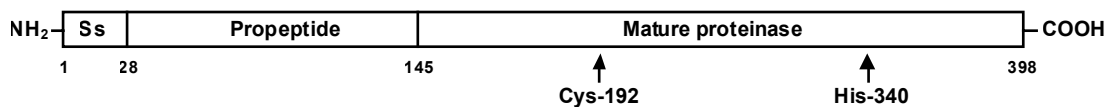


Figure 1. Schematic map of SpeB

Regulation of cysteine proteinase activity and zymogen processing

The process leading to an extracellular mature and proteolytically active cysteine SpeB involves several essential steps that influence both the total amount of enzyme being produced and to what extent it is enzymatically active. A summary of the steps regulating the production and enzymatic activity of SpeB is shown in figure 2.

SpeB is transcribed during early stationary phase and is down-regulated by glucose and other nutrients in the growth medium (37). Even though the expression of SpeB is influenced by nutritional factors, it has been proposed that SpeB does not contribute to the acquisition of essential nutrients (207). The global transcriptional regulator *mga* (208), the *speB*-specific *ropB* in the Rop loci (regulation of

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proteinase) (159), and *pel* (pleiotropic effect locus) have been shown to be positive regulators of *speB* expression (141). One report has described that the two-component regulatory system CsrR-CsrS represses transcription from the *speB* gene (84), but another report concludes that CsrR-CsrS is not involved in regulation of *speB* (64). Furthermore, two peptide permeases of the ABC-transporter family have been suggested to be involved in the regulation of SpeB production (205,206).

Almost all characterized extracellular proteases from Gram-positive bacteria have a propeptide located between the signal peptide and the mature protein (257). This propeptide often maintains the enzyme in an enzymatically inactive state before it is released from the cell and could also be involved in giving the correct folding of the molecule. This has also been shown for SpeB where the proregion has a unique fold and inactivation mechanism that displaces the catalytically essential His residue from the active site (114). The molecular events leading to a release of the proregion and the production of the mature proteinase are not fully understood, but it is known that purified zymogen from streptococci is partly enzymatically active and can cleave itself under reducing conditions (30). This autocatalytical processing of the zymogen molecule is rather an intermolecular than an intramolecular event, where one zymogen molecule acts on the other by a sequential processing with at least six intermediates (58). This process does not occur when *ropA*, the second gene of the Rop loci has been inactivated. This protein, RopA, both assists SpeB in translocation via the secretory pathway as well as functions as a molecular chaperon that aids the zymogen to its autocatalytically active state (159). If one speculates, RopA could be involved, possibly in cooperation with other molecules, in the removal of the propeptide blocking of the active site in the zymogen. Furthermore, when there is no cell wall-anchored M protein, the SpeB zymogen is secreted in a conformational state that does not allow autocatalytical processing (**Paper I**).

When already active, SpeB could be regulated by host proteinase inhibitors. For instance, a peptide inhibitor based on the active site of cystatin C blocked the activity efficiently (22), while H-kininogen of the same inhibitor family did not. Surprisingly, H-kininogen served as a substrate for SpeB resulting in bradykinin release (88). *S. pyogenes* also binds the broad-spectrum proteinase inhibitor α_2 -macroglobulin by a high affinity interaction with the cell wall-anchored protein GRAB (217). This complex regulates proteolytic activity of SpeB and possibly host proteases at the bacterial surface. A recent study demonstrated that when the

human serine proteinase inhibitor α_1 -protease inhibitor is S-nitrosylated by nitric oxide, it has an inhibitory effect on SpeB (173).

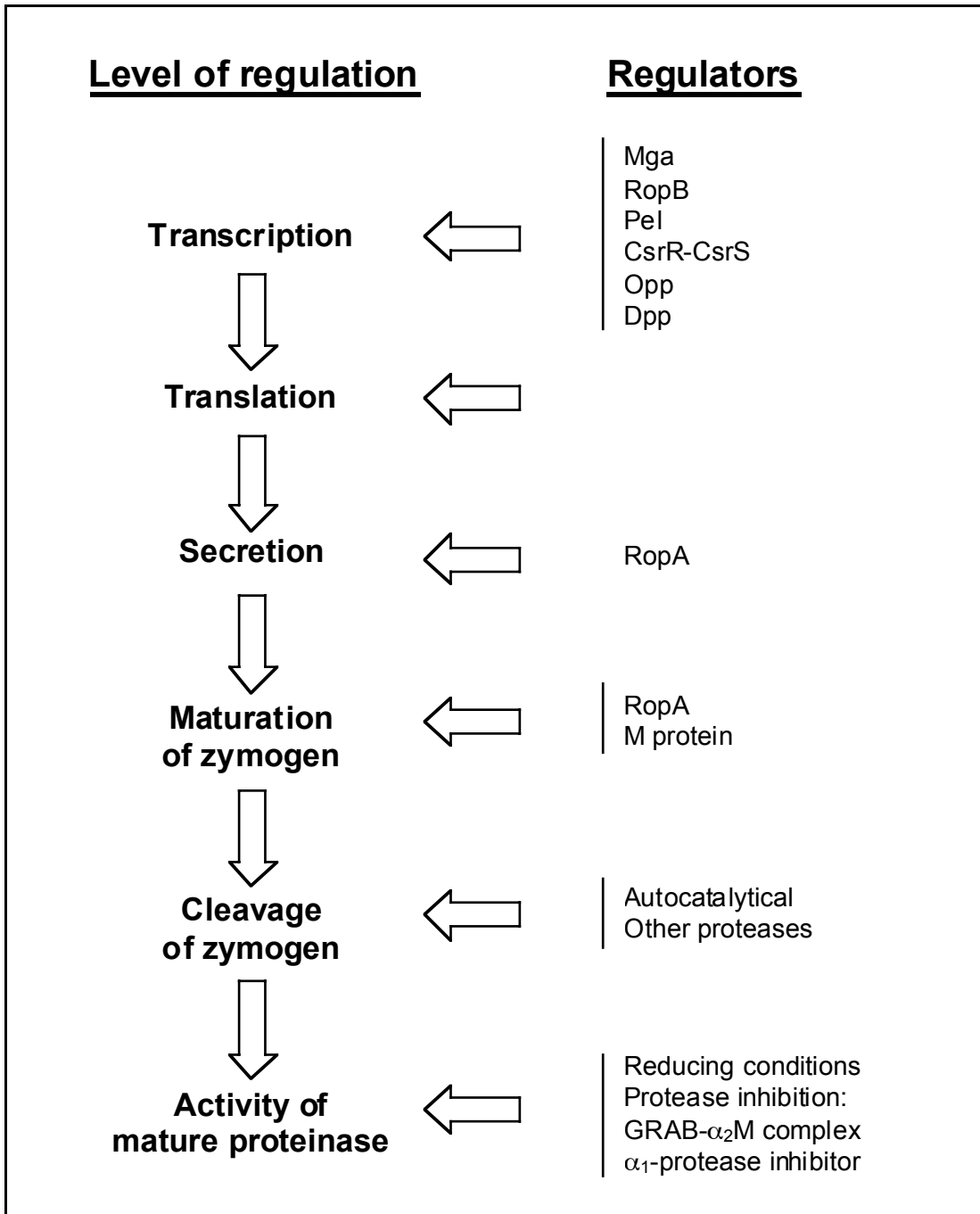


Figure 2. Regulation of SpeB activity

SpeB substrates and ligands

SpeB interacts with a number of important host molecules resulting in degradation, activation, or binding (summarized in Table II). SpeB has the ability to degrade extracellular matrix components such as fibronectin and vitronectin, which might contribute to dissemination of the bacteria in tissue (120). SpeB has recently been shown to degrade proteoglycans such as decorin with release of dermatan sulphate that inhibits antimicrobial peptides of the innate immune system (228). Furthermore, SpeB activates the host metalloprotease MMP-2, which is involved in degradation and remodeling of the ECM (27). SpeB is also active on components of the coagulation system, degrading human fibrinogen (165) and releasing active fragments from the urokinase receptor on monocytic cells (266).

SpeB can also activate and release pro-inflammatory molecules that could be important for the symptoms seen in *S. pyogenes* infections. For instance, the cytokine precursor IL-1 β is cleaved by SpeB to an active IL-1 β (119). Furthermore, SpeB has the ability to release the potent proinflammatory and vasoactive peptide bradykinin from its precursor H-kininogen (88). This release of kinins could contribute to the hypovolemic hypotension seen in sepsis caused by *S. pyogenes*. Kinin release by cysteine proteinases has also been demonstrated in the periodontitis causing bacterium *Porphyromonas gingivalis* (230) as well as in the parasitic protozoan *Trypanosoma cruzi* (54).

Beside its activities on host molecules, SpeB is also active on proteins produced by *S. pyogenes*. This was already suggested by Elliot in the 1940's, who discovered that the proteolytic activity in the bacterial supernatants decreased the number of M protein molecules on the bacterial surface (61). This proteolytic activity made serological analysis difficult due to release of the M antigen. It has now been demonstrated that purified SpeB releases biologically active fragments from both the cell wall-anchored M1 protein and C5a-peptidase (15). This was confirmed by the observation that SpeB cleavage of the M protein leads to an alteration of the bacterial IgG-binding properties (213). Furthermore, SpeB has been shown to release the cell wall-anchored collagen-like protein SclB from the bacteria (215). In addition to this activity on cell wall-anchored proteins, SpeB can cleave the secreted pore-forming lysin streptolysin O that remains cytolytically active after SpeB-processing (199).

SpeB has also been demonstrated to be involved in specific binding of several host molecules. A naturally occurring variant of SpeB contains the amino acid motif Arg-Gly-Asp (RGD) that is essential for the ligand recognition by many

human integrins; heterodimeric membrane proteins involved in numerous cell-cell and cell matrix interactions (96,224). This SpeB variant is mainly associated with the clinically important M1 serotype of *S. pyogenes* and has been shown to preferentially bind host cell integrins $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ via its RGD motif (241). Furthermore, SpeB has recently been implicated in the binding of host glycoproteins such as laminin, thyroglobulin, mucins, and fetuin (100). SpeB also functions as a superantigen stimulating T-lymphocytes without antigen presentation, and this activity is independent of the proteolytic activity (62,140).

Table II. Interactions between SpeB and host molecules

Host molecule	Activity	Reference
Fibrinogen	Degradation	(61,165)
Vitronectin	Degradation	(120)
Fibronectin	Degradation	(120)
H-kininogen	Release/activation	(88)
Interleukin 1 β	Activation	(119)
Metalloprotease MMP-2	Activation	(27)
Decorin	Degradation	(228)
Integrins	Binding	(241)
Laminin	Binding	(100)
IgG	Cleavage	Paper III
IgA, IgM, IgD and IgE	Degradation	Paper IV

SpeB as a virulence factor

Several experimental studies *in vitro* and *in vivo*, as well as clinical studies have suggested a role for SpeB as an important virulence factor. For instance, patients with invasive disease caused by different serotypes of *S. pyogenes* seroconverted to SpeB, indicating that SpeB is expressed *in vivo* during infection in humans (80). On the other hand, Holm and coworkers showed that patients with severe invasive disease had low antibody titers against SpeB. This suggests that an inability to produce SpeB-specific antibodies contributes to the development of the serious conditions caused by *S. pyogenes* (91). Furthermore, Talkington and co-workers showed that isolates of M1 serotype from TSS patients were associated with SpeB production (249). In contrast, Kansal and colleagues showed that there is an

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inverse relationship between SpeB production and disease severity; strains of the M1 serotype that produce low amounts of SpeB are more often associated with severe invasive infections than SpeB-positive strains, possibly due to a sparing of the M protein on the surface (116). Moreover, Reader and colleagues noticed absence of SpeB production in virulent large capsular forms after passage of a strain of M64 serotype in human blood and suggested that SpeB is down-regulated in human blood (212). In contrast, mice that were passively immunized with SpeB were protected against challenge with *S. pyogenes* bacteria of different serotypes (118), and mice were rescued from lethal bacterial injections by synthetic peptide that inhibits cysteine proteinase activity (22). SpeB has been shown to act synergistically with cell wall antigens and streptolysin O to induce lung injury in rats, measured by pulmonary vascular leakage, neutrophil accumulation, and cytokine elevation (232).

Several studies have revealed effects with relevance for pathogenicity in strains where the *speB* genes have been inactivated. SpeB production does not affect bacterial viability *in vitro* (36), but a number of studies have shown that the enzyme is important for the balance of the host-parasite interaction. For example, isogenic *speB* mutant strains are significantly less lethal to mice when challenged intraperitoneally (157), and caused less mortality and tissue damage when mice were infected subcutaneously (134,154). Furthermore, bacteria lacking SpeB are less resistant to phagocytosis and do not disseminate into internal organs as the wild type bacteria (152). SpeB has also been shown to play a role in host-tissue tropism, since SpeB activity increases the bacterial reproduction in an impetigo model. Moreover, epidemiological evidence suggests a correlation between SpeB production and a genetic marker for preferred tissue site of infection at the skin (246).

Somewhat conflicting conclusions have been drawn when investigating the involvement of SpeB in cellular invasion. Tsai and colleagues showed that SpeB did not influence the ability of bacteria to adhere to respiratory epithelial cells, but enhanced the frequency of bacterial invasion into the cells (253). Furthermore, they showed that SpeB induces cell death by apoptosis in the same epithelial cell line (254). On the other hand, Chaussee and coworkers showed that SpeB activity abrogates fibronectin-mediated internalization into epithelial cells (35), and Musser and co-workers showed that inactivation of SpeB enhances bacterial internalization by human epithelial and endothelial cells *in vitro* (26).

Even though there are conflicting reports of the importance of SpeB as a virulence factor, it is clear that this enzyme needs to be taken into account when discussing the various aspects of the interaction between *S. pyogenes* and the human host.

Other proteases

The C5a peptidase

The C5a peptidase is a cell wall-anchored 130 kDa serine endopeptidase that specifically cleaves the complement factor C5a (38,42,262). The group A streptococcal gene encoding C5a peptidase, *scpA*, is very similar to the group B streptococcal *scpB* gene suggesting horizontal gene transfer between the species (39). By cleaving the chemotactic complement factor C5a, recruitment of phagocytic cells to the infectious site is inhibited (110). C5a is important in activating neutrophils that phagocytize *S. pyogenes* (111). Intranasal immunization with C5a peptidase has been shown to prevent nasopharyngeal colonization of mice by *S. pyogenes* suggesting a role for the C5a peptidase as a colonization factor (109). Furthermore, in a mouse model of long-term colonization, an *S. pyogenes* strain where the *scpA* gene was deleted caused pneumonia to a lower frequency than wild type bacteria (95).

Serum opacity factor

The ability of *S. pyogenes* to induce opacity of human serum is well established. The protein responsible for this reaction is serum opacity factor (SOF). Antibodies against SOF inhibit the opacity reaction, and are used for typing of *S. pyogenes*, since the antigenic property of SOF correlates with the M serotype (168,264). The purified enzyme has been characterized and shown to be an apolipoproteinase that cleaves the A1 component of high-density lipoproteins in serum (226,227). When the gene encoding SOF was sequenced, a fibronectin-binding repeat domain was apparent (214). Several variants of SOF have been identified in different strains and the fibronectin-binding capacity of SOF-expressing strains contributes to virulence in a mouse model of infection (44,131,132).

Enzymes interacting with carbohydrates

The surface associated proteins SDH and SEN are both glycolytic enzymes. SDH, a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is found in the majority of *S. pyogenes* isolates and it binds a number of human proteins such as lysozyme, fibronectin, as well as the cytoskeletal proteins actin and myosin (192). In addition, SDH ADP-ribosylates itself in the presence of NAD. This ADP-ribosylating activity is stimulated by nitric oxide, and ADP-ribosylated SDH loses its GAPDH activity (193). Furthermore, SDH is involved in signal transduction between the bacteria and pharyngeal cells. SDH binds specifically to a human cell membrane protein and activates both tyrosine kinases and protein kinase C, ultimately leading to activation of histone H3-specific kinases (194).

SEN is an α -enolase that converts phosphoglycerate to phosphoenolpyruvate, but the mainly studied property is its ability to interact with human plasminogen. The acquired plasmin activity on the streptococcal surface is thought to play a role in bacterial tissue invasion (195). Furthermore, antibodies against SEN are known to cross-react with human α -enolases, and could thereby contribute to the development of autoimmune diseases following streptococcal infections (69).

It was discovered already in 1941 that *S. pyogenes* and other pathogenic streptococci were capable of degrading hyaluronic acid (170). This hyaluronidase cleaves hyaluronic acid, a sugar polymer composed of alternating *N*-acetylglucosamine and glucuronic acid residues. Hyaluronidases have been suggested to contribute to the spreading of bacteria in tissue since hyaluronic acid is a major ECM component in different tissues. Alternatively, these enzymes are believed to remodel the bacterial capsule that is composed solely of hyaluronic acid (170). Most of the described hyaluronidases from *S. pyogenes* are encoded by temperate bacteriophages, and the presence of collagen-like repeats in the protein has been suggested to contribute to the generation of antibodies cross-reacting with collagen and induction of the polyarthritis seen in RA (97-99).

Another secreted enzyme is the neuraminidase, which releases terminal sialic acids from glycoproteins including immunoglobulins, and is associated with strains causing APSGN (51,83,177). This neuraminidase will be further discussed in the chapter “**Microbial enzymes interacting with immunoglobulins**”.

PHOSPHOTRANSFERASE SYSTEMS

Introduction

Bacterial phosphotransferase systems (PTS) are involved in the transport of carbohydrates across the cellular membrane and in phosphorylation of the imported carbohydrates. PTS also work as signaling pathways that regulate gene expression in response to environmental carbohydrate content. PTS have been extensively studied in Gram-negative bacteria, but have also been identified and characterized in Gram-positives (225). All PTS are built up of a membrane-spanning receptor/pore complex and two intracellular soluble proteins, Enzyme I (EI) and Histidine Protein (HPr). EI and HPr participate in the phosphorylation of all PTS carbohydrates and are therefore referred to as the general PTS proteins. A schematic drawing of a typical PTS is shown in figure 3.

The specificity of each PTS is conferred by the carbohydrate receptor/pore-complex, consisting of the carbohydrate-specific Enzyme II (EII). EII may be composed of a single membrane-bound protein comprising three domains (A, B, and C), or two or more proteins of which at least one is membrane-bound (B and C). In either case, the phospho-group is transferred from intracellular phosphoenolpyruvate (PEP) to the carbohydrate via obligatory phospho-intermediates of EI, HPr, EIIA, and EIIB. A 16 kDa HPr component has been identified in *S. pyogenes* (56), and this protein is recognized by antibodies generated against HPr from *S. sanguis* (107).

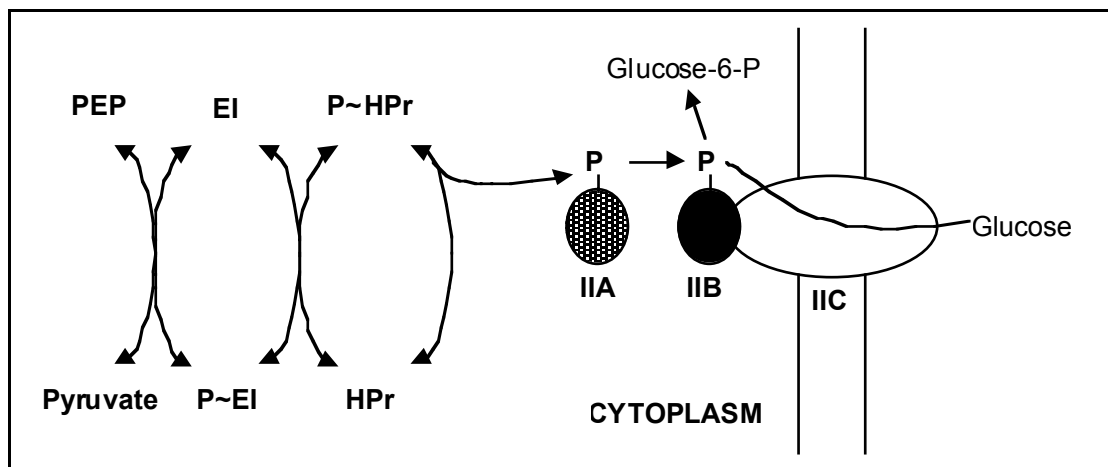


Figure 3. Schematic representation of a phosphotransferase system

Catabolite repression mediated via PTS

Beside transport and phosphorylation of carbohydrates, PTS function as signal transduction systems in response to carbohydrate levels in the environment. This has been extensively studied in *Escherichia coli*, but also in Gram-positives such as *Bacillus subtilis* and *Lactococcus lactis* (225). This is referred to as catabolite repression since genes involved in the catabolism of carbon sources, but also other genes, are down-regulated in response to PTS carbohydrates above certain thresholds (34). The key player in the signal transduction system is HPr that is phosphorylated at a conserved histidine residue to varying degree depending on the equilibrium in the phosphate transfer from PEP via EI and HPr to EII. The phosphorylation state of HPr determines its activity towards the catabolite control protein CcpA, a DNA-binding protein that binds to catabolite response elements, *cre*, in the promoter region of catabolite-controlled genes (183).

In *S. pyogenes*, a glucose-induced PTS was identified by RNA fingerprinting, and the genes encoding the general components HPr, EI, and CcpA could be identified by searches against the SGSP database (**Paper II**).

In addition to the PEP-dependent histidyl-phosphorylation, there is an ATP-dependent kinase, which seryl-phosphorylates HPr in response to glycolytic intermediates (218). This seryl-phosphorylated HPr cannot transfer its phosphate to sugars via EII, but instead depends on phosphatase activity. These two pathways of HPr phosphorylation are coordinately regulated since the phosphatase activity is strongly inhibited by PEP and EI (56). No further analysis of *S. pyogenes* catabolite repression has been reported but several studies on *L. lactis*, *Streptococcus mutans*, and *B. subtilis* have shown the importance of HPr and CcpA in catabolite repression (150,151). Furthermore, the global effects of CcpA as a transcription factor have been studied in *B. subtilis* using a whole-genome approach (176). Also in *Enterococcus faecalis* the effects of inactivation of the *ccpA* gene was studied using two-dimensional protein electrophoresis and phosphorylation analysis of HPr, confirming that CcpA is a catabolite control protein (138).

HUMAN IMMUNOGLOBULINS

The human defense mechanisms against invading microorganisms can be divided in the innate and acquired immune systems. The components of the innate, or natural, immune defense are present prior to exposure of infectious microbes or other foreign macromolecules, are not enhanced by exposure, and do not

discriminate among foreign substances. In contrast, the acquired or specific immune system, is stimulated by exposure to foreign macromolecules, is specific for distinct macromolecules, and increases the defensive capability after repeated exposure to the same foreign molecule or microbe.

The first line of defense against invading microorganisms is the physiochemical barrier lining the outer surface of the body. Most of these barriers including the skin, the mucous membranes in the respiratory, gastrointestinal, and urogenital tracts could be considered parts of the innate immune system. However, secretions from these surfaces contain molecules, such as antibodies, from the acquired immune system. Other constituents of the immune defense are the circulating molecules specialized for interactions with foreign substances. These molecules include the complement system and the antibodies, or immunoglobulins (Igs). The complement system could be regarded as one of the cross-bridges between the innate and acquired immune defense, since it can be activated either directly by foreign (cell) surfaces that lacks the appropriate inhibitors (alternative pathway), or via antibodies bound to specific antigens on foreign surfaces (classical pathway).

The Igs are expressed by specialized white blood cells of the acquired immune system, B-lymphocytes (B-cells), produced in the bone marrow. The Igs produced by the B-cells are specific for epitopes on antigen molecules such as polypeptides, polysaccharides, or soluble lipids. B-cell diversity arises during development of the immune system, exists before exposure to foreign antigens and the repertoire of epitopes B-cells recognize is extremely large. Clones of B-cells encountering a foreign antigen will expand and the B-cells proliferate and mature into plasma cells that produce large amounts of immunoglobulins recognizing the antigen. These antibodies bind to for instance surface structures on a bacterium. This "labeling" or opsonization of the bacterium enhances complement activation with release of chemotactic substances such as C5a that recruits phagocytic cells with specific antibody receptors. This could lead to a direct killing of the bacterium by complement through assembly of MAC into the membrane, or by engulfment of the bacterium by phagocytic cells such as macrophages or neutrophils. Phagocytosis by these cells occurs even without antibodies, but the opsonization and Fc receptor binding enhances the process dramatically. Thus, antibodies serve to direct and amplify the defense mechanisms against a recognized invading microorganism.

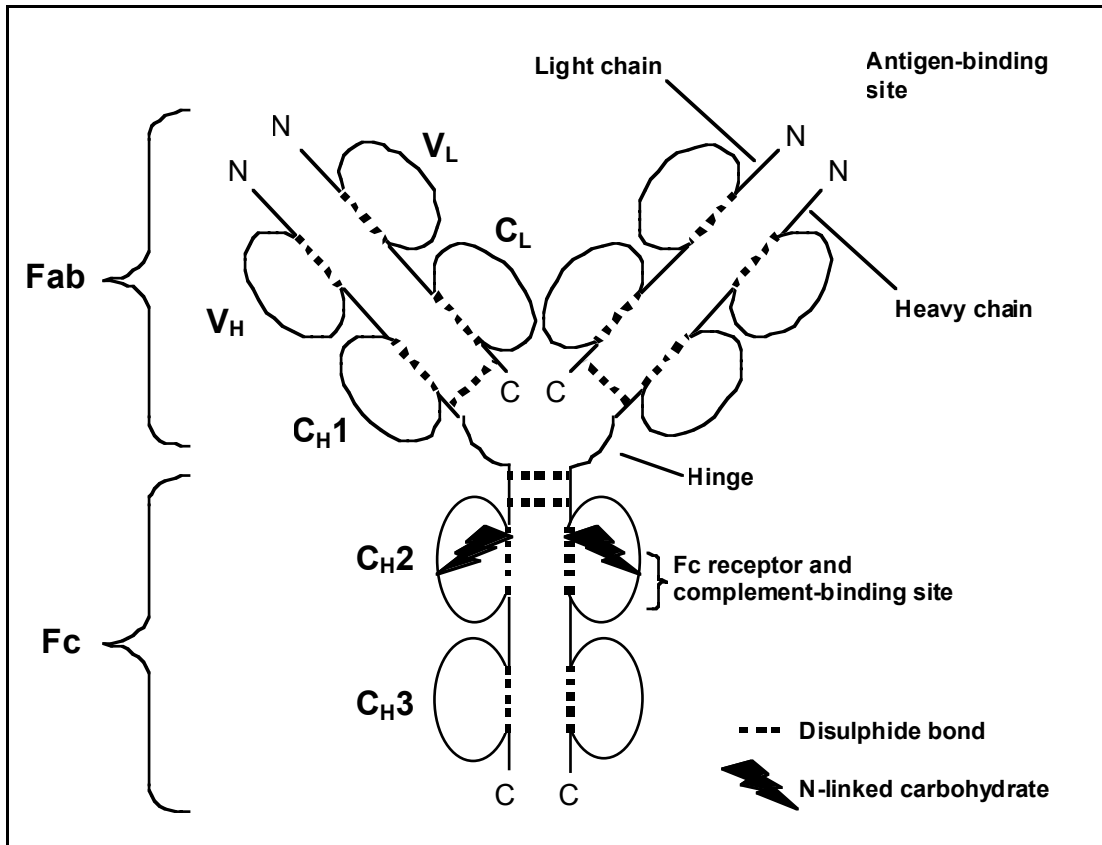


Figure 3. Schematic diagram of an IgG molecule

Structure

All antibodies are very similar in their overall structure, with two identical light chain of about 24 kDa and two identical heavy chains of 50-70 kDa. A series of repeated units about 110 amino acids can be found in both the light and heavy chains. Each repeat independently forms the typical immunoglobulin fold with two layers of β -pleated sheets with three or four strands of antiparallel polypeptide chains held together by a disulphide bond. This structural unit is a common motif in number of proteins of the Ig-superfamily, including the T-lymphocyte receptor, the antigen presenting major histocompatibility complexes (MHC), Fc receptors, cellular adhesion molecules, cytokine receptors, and growth factor receptors (94). Despite the overall similarities, the human immunoglobulins can be divided into the isotype classes IgA, IgD, IgE, IgG, and IgM, based on differences in size and amino acid composition of the heavy chains. IgG can be further divided into subclasses IgG1 to IgG4 and IgA into subclasses IgA1 and IgA2. The differences in the heavy chains designated with the Greek letters α , δ , ϵ , γ and μ , define the effector function of the corresponding iso- or subtype. Thus, the heavy chains bind differently to Fc

receptors, complement factors, and cell surface receptors. The two different light chains κ and λ do not influence the effector function of the antibodies.

The heavy chain contains three constant domains C_{H1-3} and one variable domain V_H , and the light chain contains one variable, V_L , and one constant domain C_L . The antigen-binding part of the Ig molecule is constituted by the V_L and V_H domains (See figure 3). These portions of the molecule accounts for the antigen-specificity (Fab), and is separated from the effector triggering part (Fc) of the molecule by the flexible hinge region. The hinge region provides flexibility to the Ig, but the disadvantage is that it also is an excellent target for proteases produced by pathogenic microorganisms (see **Microbial enzymes interacting with immunoglobulins**). This protease-sensitive region makes it possible to separate the molecule into defined fragments whose biological activity and structure then can be studied independently (265). For instance, papain cleaves Ig in the hinge region NH_2 -terminally of the disulphide bonds holding the heavy chains together. This results in three Ig fragments, two monomeric antigen-binding Fab fragments, and one Fc fragment (209). In contrast, pepsin cleaves between the two disulphide bonds releasing a dimeric antigen-binding fragment, $F(ab')_2$, and degrades the Fc portion into low molecular weight fragments (29).

Immunoglobulin functions

The effector functions of antibodies are triggered by antigen-binding and most of their effects depend on the characteristics of the Fc portions of the different iso- or subtypes of Igs. Some functions though can be performed by any Ig isotype or isolated Fab or $F(ab')_2$ fragments. These can sterically hinder foreign toxins, bacteria, viruses from binding to receptor or other cellular structures, and thereby neutralize the toxic or infectious process. Furthermore, soluble enzymes can be inhibited by antibodies directed towards for instance the catalytic domain of the enzyme. For example, it has been shown that such neutralizing antibodies can inhibit the activity of the streptococcal cysteine proteinase, SpeB (165).

A resting B-cell that recognizes an antigen by its membrane-bound Ig is stimulated to proliferate and produce Ig of that particular idotype with the hypervariable regions of heavy and light chains directed against the antigen. The heavy chain of the membrane-bound Ig differs from the soluble form in that it contains an extra COOH-terminal hydrophobic domain that spans the cellular membrane. During its life span, the B-cell can produce different heavy chain

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isotypes. For example, immature B-cells may express IgM, previously unstimulated mature B-cells may express IgM and IgD, and previously stimulated memory B-cells may express any isotype or subtype. This class switching allows the production of antibodies recognizing the same antigen, but with different effector functions (234).

Complement activation

The complement system consists of a family of serum and membrane-bound proteins that can be activated by a proteolytic cascade that generates effector molecules. Complement mediates many of the cytolytic and inflammatory effects of humoral immunity. The classical pathway of complement activation is triggered when C1_q binds to the Fc region of IgG or IgM complexed to an antigen. C1_q binds to the single C_γ2 domain of IgG, but requires clustering of IgG bound to for instance antigens on a surface, or to aggregated IgG in antibody-antigen complexes. The different subclasses of IgG vary in their ability to activate complement. IgG3 is the most efficient complement activator, IgG1 is somewhat less efficient, IgG2 is a poor activator, and IgG4 does not activate complement at all (265).

Complement activation by the pentameric IgM molecule is sterically hindered when in solution, but when bound to a surface, C1_q is allowed to interact with the Fc portion via the C_μ3 domain.

Opsonization and phagocytosis

The process of phagocytosis of foreign material by for instance neutrophils or macrophages can occur independently of immunoglobulins, but the process is markedly enhanced if the particle to be ingested is covered by IgG bound via Fab to the surface. This process, termed opsonization, recruits phagocytic cells with receptors recognizing the Fc-portion of IgG (Fc_γR). There are three main types of Fc_γRs, and IgG1 and IgG3 are the most efficient subtypes in phagocytosis promotion. Besides serving as a surface marker on the foreign material, Fc_γR-mediated signaling increases the efficiency of intracellular degradation of the ingested matter.

Antibody-dependent cell-mediated cytotoxicity

Several different types of leukocytes have the ability to lyse various types of cells that are coated with specific IgG. This is called antibody-dependent cell-mediated cytotoxicity (ADCC). These cells have low affinity Fc_γRs on their surface that

directs them to preferentially kill IgG-coated cells, and they do not to bind IgG in solution. Furthermore, upon contact between IgG and Fc γ R, on for instance a natural killer cell (NK), synthesis and release of cytokines like tumor necrosis factor (TNF) and interferon- γ is triggered. This together with the discharge of the cells granules are thought to mediate the cytolytic process. ADCC directed against certain kinds of parasites is mediated by IgE, or in some cases IgA, that is recognized by FcRs on eosinophils.

Mucosal immunity

IgA is the only immunoglobulin that can be selectively transported across mucosal barriers. Lining the mucosa are epithelial cells expressing Fc α Rs on their basal surfaces facing the blood stream. Upon binding, IgA is transferred to the mucosal surface by vesicular transport. The dimeric IgA molecule is then released together with a part of the receptor (secretory piece) by specific cleavage. IgA on mucosal surfaces plays an important role in the defense against respiratory and intestinal pathogens. Furthermore, secretory IgA is present in cutaneous secretions defending the organisms against infections in the skin.

Glycosylation of immunoglobulins

A large number of molecules in the immune system are glycoproteins that are functionally dependent on their glycosylation pattern (222). This is also true for Ig, where the different isotypes are glycosylated to varying extent. In IgG, there is a conserved N-glycosylation site at Asn-297 in the C γ 2 domain (128). In addition, some reports have suggested that IgG from myelomas are N-glycosylated at various sites in the Fab region (127). The two conserved Fc glycans do not extend into solvent, but forms a bridge between the two opposing C γ 2 domain (243). The conserved glycan on IgG is a complex biantennary structure with a core fucose and bisecting GlcNAc (se figure 1 in paper III). There are several naturally occurring variants of this glycan with varying degree of sialylation and galactosylation as well as absence of bisecting GlcNAc (60). Glycans without terminal sialic acid and lacking both galactose residues are termed G0, glycans lacking one galactose G1, and fully galactosylated glycans G2 (211). The composition of this glycan has been shown to be important for the functional properties of IgG. For instance, murine IgG completely lacking the oligosaccharide does not activate complement, does not bind to Fc receptors on macrophages, and antibody-antigen complexes stay in the

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circulation for longer time than normal IgG (186). Furthermore, expression of human monoclonal chimeric antibodies in mice revealed that IgG1 with truncated oligosaccharides cannot bind C1_q or induce complement-mediated hemolysis, but has only slightly reduced affinity for Fc γ RI (269). Complete removal of the oligosaccharide from IgG1 dramatically decreases the binding to Fc γ RIII (210). Furthermore, it has been shown that noncovalent interactions between the primary GlcNAc and Asp-265 of the γ -chain are essential for recognition of IgG3 by Fc γ RI and Fc γ RII (158).

In human IgM, there are five N-glycosylation sites on the μ -chain, but the structure and carbohydrate composition of the attached oligosaccharides have only been described for parts of the molecule (268). The α -chain of IgA1 also contains two N-glycosylation sites to which highly sialylated and galactosylated oligosaccharides are attached (10). In addition, IgA1 is both N- and O-glycosylated in the Fab region (167). In contrast to IgG, IgA and IgD also contain O-linked oligosaccharides in the hinge region, which could partly protect this region from proteolysis (67,74).

IgG glycosylation abnormalities and disease

The glycosylation state of IgG is important for a number of pathophysiological conditions, mostly autoimmune diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) with Sjögren's syndrome, and inflammatory bowel diseases (IBD) including Crohn's disease (59,197). Agalactosyl IgG glycans, G0, have also been detected in mycobacterial tuberculosis patients (196). For a recent review on glycosylation and rheumatic disease, see (8).

A diagnostic feature of RA is the rheumatoid factor (RF), antibodies that recognizes the Fc portion of IgG (256). RFs can be detected early in the disease process of RA and are used clinically to predict the disease (55). RFs are not exclusively found in RA patients, but also in mixed essential cryoglobulinemia (MEC), where high levels of circulating RFs can be found, and many of the studies on RFs have been using such purified RFs (6,79). The most studied RFs are of IgM isotype, but IgG and IgA RFs have also been studied. RFs bind to the Fc region of IgG in the C_H2-C_H3 interface close to the binding site for the streptococcal and staphylococcal proteins G and A (190,242). It has been shown that the autoantibody activity of RF increases with decreasing levels of galactosylation of the N-linked glycan on IgG (166,182). Furthermore, nonpathogenic levels of antibodies against collagen were made pathogenic in murine collagen-induced arthritis by removing

the terminal galactoses on the glycan (211). The agalactosyl antibodies (G0) associated with RA can also interact with the lectin mannose-binding protein (MBP). This interaction leads to activation of the complement system that could contribute to the chronic inflammation of the synovial membrane in RA (162). The production of agalactosyl IgG in RA patients is due to impaired galactosyltransferase activity in their B-cells (9), and isolated B-cells from RA patients produce agalactosyl IgG (23).

Another feature of agalactosyl IgG is that these proteins have a tendency to aggregate and form circulating complexes easier than normal IgG. It has been shown that murine IgG lacking the glycan completely, or IgG-G0, is cleared much slower from the circulation than normal IgG (181,186). In several autoimmune disorders including RA and SLE, these complexes of for instance RF and other antibodies precipitate at low temperatures, and are thus called cryoglobulins (24). Cryoglobulins have been implicated in the development of renal, vascular, and neurological complications of autoimmune diseases. It has been shown that the glycosylation of IgG is important for the formation of pathogenic cryoglobulins. Agalactosyl antibodies have been shown to form cryoglobulins much easier than fully galactosylated IgG and induced severe lupus-like glomerulonephritis in a mouse model (174).

Bacterial interactions with immunoglobulins

Immunoglobulin-binding proteins

Specific binding of immunoglobulins via cell wall-anchored proteins is a common feature of Gram-positive bacteria such as *S. aureus* and streptococci of groups A, C, and G (21,70). In *S. pyogenes*, the M- and M-like proteins bind human IgG via the Fc-portion in a non-immune fashion (3,85,219). Several functions for these interactions have been suggested including molecular mimicry, nutrition, adherence, and environmental sensing (41). Protein H of *S. pyogenes* and protein A of *S. aureus* inhibit complement deposition and activation on IgG-coated targets *in vitro* (16,198). Furthermore, protein H is sufficient for bacterial survival in human blood in the absence of cell wall-anchored M1 protein (122). Both these proteins, protein A and H, as well as the group C and G streptococcal protein G, bind to closely related sites in the C γ 2-C γ 3 interface of the Fc region (72). Both protein A

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and protein H binding to IgG is independent of an intact glycan on Asn-297 in the γ -chain (137,186) and (**Paper III**).

In addition to the IgG-binding proteins, both group A and B streptococci express IgA-binding proteins (86,143). The *S. pyogenes* IgA-binding protein Arp4 has extensive sequence similarity to M proteins (75) and it mainly binds IgA, while Sir22 binds both IgA and IgG (237). Sir22 interacts with the C α 2-C α 3 interdomain region of IgA. Furthermore, Sir22 inhibits the interaction between IgA and the Fc α R CD89 and thereby interferes with IgA effector functions (204).

Furthermore, beside the cell wall-anchored Ig-binding proteins, a recently identified secreted protein, SibA, binds all subclasses of IgG as well as IgA and IgM. The overall similarity with M proteins is low, but SibA has a predicted α -helical structure that could be involved in the binding of Ig (63).

Microbial enzymes interacting with immunoglobulins

A number of enzymatic interactions between microbial pathogens and human immunoglobulins are thought to contribute to pathogenesis. For instance, proteases capable of cleaving the hinge region of human IgA have been extensively studied. As previously discussed, the flexible hinge region of IgA1 is protected from proteolysis by multiple O-linked glycans (167). Nevertheless, several pathogens have evolved specific IgA-proteases that cleave at specific sites in the hinge region of IgA [for a review see (125)]. The first examples of IgA-proteases were described in *S. sanguis* and *Neisseria* species in the mid-1970s (200). Subsequently, IgA-proteases have been described for a number of bacterial species that colonize or infect the mucosal membranes of humans, such as oral streptococci, (123), *Haemophilus influenzae* and *S. pneumoniae* (124,161). As a result of the specific activity of these IgA-proteases, the IgA molecule is cleaved into a stable Fc fragment and two monomeric Fab fragments that retain their antigen-binding capacity (163,164). One of the IgA subclasses, IgA2 is more resistant against proteolysis due to lack of a specific peptide stretch that can be found in the hinge region of IgA1 (203). The IgA-proteases have been shown to destroy the activity of IgA (202), and IgA-protease production distinguishes pathogenic from harmless *Neisseria* (178). No specific IgA-protease has been identified in *S. pyogenes*, but the streptococcal cysteine proteinase SpeB degrades the COOH-terminal part of the IgA molecule (**Paper IV**).

Microbial proteases interacting with IgG have not been as extensively studied as the IgA-proteases. However, a number of pathogens produce IgG-

degrading proteases. These include the oral pathogens *Prevotella intermedia* and *Prevotella nigrescens* isolated from periodontal pockets and oral abscesses. Inhibition experiments suggest that these pathogens degrade IgG due to cysteine proteinase activity (104). In addition, a *Pseudomonas aeruginosa* elastase implicated as a virulence factor degrades human IgG *in vitro*, and its activity could be inhibited by local treatment with the protease inhibitor α_2M (89). Furthermore, a secreted cysteine proteinase from the helminth parasite *Paragonimus westermani* attenuated the effector functions of human eosinophils stimulated with IgG (235), and proteases from *Serratia marcescens* cleave both IgG and IgA around the hinge region (175).

In *S. pyogenes*, SpeB cleaves IgG in a manner similar to the IgA-proteases; thus, IgG is cleaved at a defined site in the hinge region into two stable monomeric Fab fragments and one Fc fragment (**Paper III**). This IgG-cleavage by SpeB significantly reduced the capacity of opsonizing IgG to kill *S. pyogenes* in human blood (**Paper V**). Furthermore, SpeB degraded the COOH-terminal parts of the heavy chains of IgA, IgM, and IgD into low molecular weight fragments, while the heavy chains of IgE were completely degraded (**Paper IV**).

Apart from enzymes cleaving or degrading the peptide backbone of IgG, enzymes hydrolyzing the conserved N-linked glycan have been implicated in the pathogenicity of *S. pyogenes*. An extracellular neuraminidase activity was first described to release sialic acid from bovine submaxillary mucins (51,83). Strains isolated from patients with APSGN were shown to produce neuraminidase activity that releases terminal sialic acids from the glycans on human IgM, IgG, fibrinogen, and renal basement membranes. The alterations of the immunoglobulins were suggested to play a role in the development of APSGN, since all the nephritogenic, but none of the rheumatogenic strains tested, expressed neuraminidase activity (177). To date, no *S. pyogenes* neuraminidase gene has been described. The recently identified endoglycosidase, EndoS, has a more drastic effect on the glycan of IgG. It hydrolyzes the chitibiose core and leaves the innermost GlcNAc with an attached fucose on the peptide backbone (**Paper III**). EndoS is active on native, but not on denatured IgG (**Paper IV**). Furthermore, opsonizing IgG treated with EndoS is significantly impaired in its ability to kill bacteria in human blood (**Paper V**).

PRESENT INVESTIGATION

Maturation of the cysteine proteinase (Paper I)

The streptococcal cysteine proteinase, SpeB, is an extensively studied secreted proteinase from *S. pyogenes* with a number of important substrates in human plasma and extracellular matrix. To obtain an active extracellular enzyme, several events have to occur. Secretion of the zymogen by means of the signal peptide, removal of the signal peptide by a signal peptidase, proteolytic cleavage of the 40 kDa zymogen into mature 28 kDa proteinase, and finally reduction of the thiol group on the cysteine residue in the active site. Cleavage of the zymogen can be obtained either by an exogenous protease or by an autocatalytic event where one zymogen molecule can act on the other (58,146).

In this study, we show that in this autocatalytic event does not take place when the zymogen is in an abnormal conformational state. Furthermore, we show that the unfolding of the zymogen could not occur unless the M1 protein was attached to the cell wall of the bacteria. This was investigated by using an isogenic M protein deficient strain generated by homologous recombination (Fig. 1). This mutant strain was subsequently analyzed for both SpeB secretion and enzymatic activity. This revealed that the mutant strain secreted approximately the same total amount of SpeB as the wild type strain, but no cleavage of the zymogen into a mature proteinase could be detected (Fig. 6). Furthermore, the mutant strain did not produce any proteolytic activity against a chromogenic substrate (Fig. 5). This suggests that the M protein is involved in the process of obtaining an active cysteine proteinase.

Furthermore, this zymogen molecule secreted from the mutant strain was possible to cleave with exogenous active cysteine proteinase. This indicates that the cleavage site in the proregion was accessible (Fig. 9). In contrast, extensive incubation of the zymogen under reducing conditions did not lead to conversion into the proteinase, unless urea was added to unfold the zymogen (Fig. 10). This suggests that the zymogen has a conformation where the active site is blocked from activity on neighboring molecules. This difference in conformation was confirmed by comparative electrophoresis of the native zymogen from wild type and mutant. This showed that the zymogen from the mutant migrated significantly faster in gel matrix, arguing for a more compact fold than the wild type protein (Fig. 11).

Taken together, our data indicate that the M1 protein at the bacterial surface acts as a protease maturation protein or an extracellular chaperone that aids the

zymogen, either directly or indirectly, into the proper conformational state. This is analogous to the *Lactococcus lactis* system, where a surface lipoprotein, PrtM, is required for maturation and enzymatic activity of a secreted serine proteinase (255).

Analysis of differential gene expression (Paper II)

In order to understand how bacteria adapt to different environments, various techniques have been developed to analyze what genes are expressed under different conditions. One such technique is RNA fingerprinting by arbitrarily primed RT-PCR (RAP) that has been widely used in eukaryotic organisms (171), but also on Gram-positive-, Gram-negative-, and mycobacteria (220,233,267,272).

To perform an RNA fingerprinting experiment, total RNA is prepared from cells grown under different environmental conditions, such as absence or presence of certain nutrients, growth factors or other molecules such as trace metals that could influence gene expression pattern. The RNA is subsequently transcribed *in vitro* into cDNA using reverse transcriptase plus an arbitrary primer at low stringency conditions. This is followed by PCR with radiolabeled nucleotides and the same arbitrary primer at low stringency for one cycle followed by 30 to 40 cycles at high stringency. The resulting products are separated on a polyacrylamide sequencing gel and visualized by autoradiography. This autoradiogram gives a representation of transcripts that makes it possible to identify genes that are differentially expressed under the given conditions. Identified bands are then excised and the DNA is eluted from the gel. This DNA can then be used as template in a second high stringency PCR with the same arbitrary primer. The resulting products can subsequently be cloned, sequenced, characterized, and identified.

In this study, RAP was applied on *S. pyogenes* in order to identify genes that are regulated in response to environmental changes. When *S. pyogenes* was cultured under glucose-rich conditions, a number of up-regulated RNA messages were identified. These products were cloned, sequenced, and identified through searches against the SGSP database (66). Among the identified products was a putative IIBC complex of a phosphotransferase system. In addition, by searching the SGSP database the genes encoding the general intracellular PTS proteins EI, HPr, and a putative catabolite control protein CcpA, were identified. Thus, RNA fingerprinting is a useful tool to identify *S. pyogenes* genes that are expressed under certain environmental conditions. Furthermore, the novel phosphotransferase

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system identified in paper III could be involved in catabolite repression of genes important for *S. pyogenes* virulence. For instance, expression of SpeB, which is down-regulated by glucose (37), could be controlled by PTS. Preliminary experiments have shown that an isogenic strain mutated in the *ccpA* gene is relieved from the catabolite repression of SpeB expression. Furthermore, there is a potential *cre* site directly upstream of the *speB* gene, arguing for CcpA-mediated regulation of this gene (Collin and Olsén, unpublished data). Future experiments will elucidate the possible role for specific and general PTS components in the regulation of *S. pyogenes* virulence genes.

A novel endoglycosidase with specific activity on human IgG (Paper III)

In *S. pyogenes*, a neuraminidase activity has been implicated in the development of APSGN, due to its ability to release terminal sialic acids from several glycoproteins including IgM and IgG (51,177). Several glycoprotein-hydrolyzing enzymes from related bacteria such as *S. pneumoniae* and *S. oralis* are used to acquire nutrients from host proteins and the enzymes have been suggested to play a role in pathogenesis (31,40).

In this study, a novel secreted 108 kDa protein from *S. pyogenes* was identified (Fig. 2). This extracellular protein was identified by NH₂-terminal sequencing and searches against the SGSP database (66,221), revealing that it is encoded by an open reading frame of 995 amino acids similar to the endo- β -*N*-acetylglucosaminidase F₂ from *Flavobacterium meningosepticum* (251). EndoF₂ belongs to the enzyme family 18 of chitinases (87). The enzymes from this family degrade chitin, *N*-acetylglucosamine polymers, and contain an amino acid motif with a glutamic acid that is crucial for enzymatic activity (260). The overall similarity between the 108 kDa protein and EndoF₂ was not particularly high, but the enzymatic motif including the glutamic acid was present in both proteins (Fig. 3). Therefore, this novel protein from *S. pyogenes* was denoted EndoS for endoglycosidase of streptococci. The corresponding gene, denoted *ndoS*, was sequenced in the strain used in this study, showing to be almost identical to the *ndoS* gene in SF370, sequenced in SGSP. In order to analyze the function of EndoS, an isogenic mutant strain lacking EndoS expression was generated by homologous recombination (Fig. 4).

Since related endoglycosidases have the ability to cleave the chitibiose core in N-linked glycans, we investigated if EndoS had any activity on standard chitinase substrates as well as N-linked host glycoproteins. This revealed that EndoS has no activity on chromogenic or fluorogenic substrates used for the analysis of other endoglycosidases such as EndoF₁₋₃. Furthermore, EndoS did not hydrolyze the heavily N-glycosylated plasma protein α_1 -acid glycoprotein known to be a substrate for related enzymes from *S. oralis* (31).

The crucial experiment in revealing the substrate specificity of EndoS was when wild type and EndoS-mutant bacteria were grown in the presence of human plasma. After growth, the plasma proteins were analyzed by SDS-PAGE. This revealed that both wild type and EndoS mutant bacteria cleaved the IgG heavy chain into fragments, but the resulting fragments differed in size by approximately 4 kDa (Fig. 5). This led to the hypothesis that both wild type and EndoS-deficient bacteria have the ability to cleave the IgG heavy chain. This could be due to the activity of the streptococcal cysteine proteinase, SpeB. SpeB is structurally related to papain known to cleave IgG into Fc and Fab fragments (114). This hypothesis was confirmed using an isogenic SpeB-mutant that did not cleave the IgG heavy chain (Fig. 6).

Furthermore, the size difference between the heavy chain fragments most likely represents an EndoS-dependent hydrolysis of the conserved N-linked glycan of the IgG heavy chain. This was confirmed using purified human IgG incubated with culture supernatants from wild type, EndoS-, and SpeB mutant bacteria under non-reducing or reducing conditions. This demonstrated that EndoS was responsible for the size shifts and that SpeB cleaved the heavy chain into stable Fc and Fab fragments (Fig. 6).

The N-linked glycan of IgG is a complex biantennary structure with terminating sialic acid and a core fucose attached to the innermost N-acetylglucosamine (Fig. 1). Since the carbohydrate composition of the glycan is known, we analyzed how this glycan was affected by EndoS, using lectins with known oligosaccharide specificity. EndoS-treated IgG was separated and probed with the mannose-specific GNL lectin, revealing that EndoS removed the mannose residues, and thus cleaves the glycan in the chitibiose core (Fig. 7). Furthermore, EndoS-treated IgG reacts with the fucose-specific lectin UEA-1, showing that the core fucose is still attached (Fig. 8). This, together with the sequence similarities with endoglycosidases, proves that EndoS is a true endoglycosidase that cleaves

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the N-linked glycan of IgG leaving one *N*-acetylglucosamine with an attached fucose on the peptide backbone.

EndoS activity could be of importance for the functionality of the IgG molecules. This is supported by several studies showing that the conserved glycan is important for structural stability, complement activation, and binding to Fc-receptors on immune cells (186,210).

Taken together, two novel enzymatic activities of *S. pyogenes* interacting with human IgG were described in this study. First a novel endoglycosidase, EndoS, that hydrolyzes the glycan of IgG, and secondly the multifunctional cysteine proteinase, SpeB, that cleaves the heavy chain of IgG into distinct Fc and Fab fragments. These two activities could be of importance in *S. pyogenes* virulence.

SpeB and EndoS act on human immunoglobulins (Paper IV)

IgA-proteases have been described for a number of bacterial pathogens such *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis* (123,124,161,201,259). These enzymes are all specific proteases that cleave IgA into Fc and Fab fragments, and have been suggested to be involved in the colonization and virulence of these pathogens (125,178). IgG-degrading microbial enzymes have not been studied extensively, but there are some examples from human pathogens including *Pseudomonas aeruginosa*, *Prevotella intermedia* and *Prevotella nigrescens* (89,104).

We have previously shown that SpeB from *S. pyogenes* cleaves IgG into stable Fc and Fab fragments (Paper III). In this report, SpeB activity on the other human immunoglobulins IgA, IgM, IgD, and IgE, was investigated. These immunoglobulins were incubated with purified SpeB, followed by SDS-PAGE and NH₂-terminal sequence analysis. This revealed that SpeB cleaves the COOH-terminal parts of the heavy chains of IgA, IgM, and IgD into low molecular weight fragments, while the heavy chains of IgE are completely degraded (Fig 1. and Table 1). Thus, SpeB has specific IgG-protease activity, and in addition, it degrades the heavy chains of other the immunoglobulin isotypes.

Ig cleavage and degradation could be of importance for the bacteria in order to circumvent the activity of the antibody-mediated immune defense, both on mucosal surfaces as well as when bacteria disseminate into deeper tissue or the blood stream.

Furthermore, we have previously observed that EndoS hydrolyzes the glycan on native IgG. This is in contrast to many of the known related enzymes whose activities are enhanced if the substrate glycoproteins are denatured making the glycan more accessible (250). Therefore, IgG was denatured to varying extent by heating prior incubation with purified recombinant EndoS. When analyzed by SDS-PAGE and lectin blot, it was revealed that EndoS activity gradually decreased when denaturation increased (Fig. 2). This suggests that not only the glycan structure on IgG, but also the tertiary structure of the molecule is important for the interaction with EndoS.

In summary, EndoS is highly specific for glycan moieties on native IgG. Furthermore, we could attribute a broad immunoglobulin-degrading activity to the streptococcal cysteine proteinase, SpeB, in addition to its activity as a specific IgG-protease. Both these activities could be of importance in the bacterial evasion of the adaptive immune response, by degradation and cleavage of the protein structure of the immunoglobulins, but also by altering the functional properties by removal of the oligosaccharides from the Fc-region of IgG.

Enzymatic strategies to evade the immune response (Paper V)

In order to survive within a host, bacterial pathogens need to overcome the counter-acting effects of the immune defense. Many different mechanisms to achieve this have been described including interference with the complement system as well as binding and hydrolysis of immunoglobulins. Several pathogens, including oral streptococci and meningococci, express proteases that specifically cleave IgA and thereby interfere with mucosal antibody-mediated immunity (126). There are also examples of microbial enzymes that degrade human IgG (89,104). IgG is a key player in the adaptive immune response and is necessary for antibody-mediated complement activation and phagocytic killing of extracellular infectious agents.

We have previously described that SpeB cleaves IgG into distinct Fc and Fab fragments, but also degrades the other immunoglobulin isotypes A, M, D and E (**Paper IV**). We speculated that this activity on immunoglobulins could disturb the functionality of the antibody-mediated defense against *S. pyogenes*.

Another way, besides proteolysis, to interfere with IgG function is to attack the conserved N-linked glycan on the heavy chain, which is crucial for several effector functions (186,210). The only example to date of this kind of strategy is

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represented by the endoglycosidase EndoS that hydrolyzes this glycan of IgG (Paper III).

In this study, we investigated if these two enzymatic activities on human IgG influenced antibody-mediated killing of *S. pyogenes* in an *ex vivo* model system. Purified opsonizing IgG was treated with either SpeB or EndoS. These antibodies were subsequently compared with intact antibodies in a bactericidal assay. Bacteria were inoculated in fresh human blood from a non-immune host with the addition of treated or un-treated opsonizing IgG. Bacterial growth was monitored over time by plating on solid medium, and the bacterial multiplication factor was calculated. This revealed that bacteria grown in the presence of treated IgG multiplied almost as rapidly as bacteria grown in blood without any opsonizing antibodies. In contrast, intact opsonizing antibodies efficiently inhibited the bacterial growth (Figures 1B and 2B).

These results indicate that the SpeB-mediated proteolysis of IgG significantly reduces the functionality of IgG. This could be an important function of SpeB contributing to its role as a virulence factor. Furthermore, the hydrolysis of the N-linked glycan leads to an impaired function of IgG, strengthening previous reports that suggest that the glycan is essential for stability and effector functions of the Fc-portion (186,210).

Moreover, an interesting hypothesis is that hydrolysis of the IgG glycan could be of importance in the development of aseptic sequelae to *S. pyogenes* infections with autoimmune components. For instance, abnormalities in IgG glycosylation have been shown in a number of autoimmune disorders such as SLE, IBD, and RA (59,197). Defects in IgG-glycosylation have also been shown to be important for the development of cryoglobulin-induced lupus-like glomerulonephritis in mice (174), and the glycan-hydrolyzing neuraminidase has been associated with *S. pyogenes* induced glomerulonephritis (177). Therefore, IgG hydrolyzed by EndoS could be of importance in the development of ARF and APSGN where autoimmunity and antibody complexes are implicated in the disease processes.

Taken together, we show that the effects of both the IgG-protease SpeB and the IgG glycan-hydrolase EndoS impair the functionality of opsonizing human IgG and thereby enhance bacterial survival in human blood. These interactions could be of importance both during the acute infection as well as in the development of aseptic sequelae with autoimmune features.

CONCLUSIONS

1. *S. pyogenes* bacteria lacking M protein secrete SpeB in a conformational state that does not allow autocatalytical processing. This demonstrates that maturation of the streptococcal cysteine proteinase, SpeB, is dependent on cell wall-anchored M protein.
2. RNA fingerprinting can be used to identify conditionally expressed genes in *S. pyogenes*. Two of the identified glucose-induced genes, encoding a sugar-transporting and signal-transducing phosphotransferase system could be involved in sugar-repression of genes involved in *S. pyogenes* virulence.
3. The secreted endoglycosidase EndoS specifically hydrolyzes the functionally important N-linked glycan of human IgG.
4. SpeB acts as a specific IgG-protease that separates the antigen-binding Fab fragment from the effector-triggering Fc fragment. Furthermore, SpeB degrades the heavy chains of the other immunoglobulin isotypes.
5. Both EndoS and SpeB activities on IgG significantly reduce the ability of antibodies to opsonize *S. pyogenes* in human blood.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Människan är ständigt koloniserad av mikroorganismer på utsidan av kroppen. Utsidan inkluderar huden och slemhinnor i luftvägarna, ögonen, mag-tarmkanalen, urinvägarna och genitalierna. De allra flesta av dessa mikrober skadar oss inte, utan är t.o.m. till nytta för oss. En del bakterier kan dock under vissa omständigheter orsaka skada lokalt på slemhinnan men också penetrera barriärerna och sprida sig till djupare liggande vävnad eller till blodbanan. Förutom de fysiska barriärerna mot främmande ämnen, såsom hud och slemhinnor, har människans immunförsvar möjlighet att bilda antikroppar som cirkulerar i blodbanan eller utsöndras på slemhinnor. Dessa antikroppar binder till kroppsfrämmande partiklar, t.ex. bakterier. Denna märkning av bakterien lockar till sig specialiserade vita blodkroppar, fagocyter, som "äter upp" (fagocyterar) och avdödar bakterien.

En bakterieart som kan orsaka sjukdom hos människan är *Streptococcus pyogenes*, som också kallas grupp A streptokocker. Dessa bakterier orsakar oftast halsfluss som är relativt enkel att behandla med penicillin. *S. pyogenes* kan också orsaka lokala hudinfektioner som svinkoppor och rosfeber. Vissa infektioner kan spridas till mjukvävnad såsom muskler och muskelskidor med kraftig vävnadsskada som följd. Bakterierna kan också ta sig vidare till blodbanan och orsaka blodförgiftning, som är ett svårbehandlat tillstånd där flera viktiga inre organ upphör att fungera. Dödligheten hos dessa patienter är 30-60% och de flesta dör inom 72-96 timmar.

Förutom de akuta tillstånden orskade av bakterien kan autoimmuna sjukdomar uppstå i efterförloppet. Akut reumatisk feber (ARF) följer på vissa luftvägsinfektioner och ger upphov till inflammationer i leder och hjärtvävnad. ARF är fortfarande den största orsaken till förvärvade hjärtfel i utvecklingsländerna. Det har föreslagits att ARF beror på att människan bildar antikroppar mot ytstrukturer på bakterierna. Förutom att känna igen bakterien, så binder dessa antikroppar också till komponenter i t.ex. hjärtat och därmed luras immunförsvaret att angripa egna organ. En annan följsjukdom, som oftast kommer efter hudinfektioner, är akut post-streptokock glomerulonefrit (APSGN). APSGN innebär en inflammation i njurens filter, glomeruli, som kan leda till att njurens funktion blir kraftigt försämrad. Man tror att APSGN utvecklas när stora komplex av antikroppar och bakteriella proteiner fastnar i njuren och där orsakar inflammation.

I denna avhandling har studerats hur *S. pyogenes* reglerar vissa av de faktorer som anses vara av betydelse för sjukdomsutvecklingen. Vidare har

undersökts hur enzymer utsöndrade av *S. pyogenes* attackerar och förstör mänskliga antikroppar.

I det första arbetet studerades ett utsöndrat enzym, streptokock cysteinproteinase, som också kallas SpeB. Detta enzym har tidigare visat sig bryta ned komponenter i mänsklig stödjevävnad och koagulationssystemet, aktivera inflammationsstimulerande molekyler samt kroppsegna enzymer som bryter ned stödjevävnad. I djurmodeller har det visat sig att om man inaktiverar produktionen av SpeB hos bakterierna, så orsakar de inte lika allvarliga infektioner. Liksom många bakteriella proteaser (protein-nedbrytande enzymer), utsöndras SpeB i en inaktiv form, zymogen, där en del av proteinet måste tas bort för att enzymet skall bli aktivt. Vi upptäckte att om bakterierna saknar ett visst ytprotein, M-protein, så tillverkas inte något aktivt SpeB. Detta beror på att SpeB zymogenet utsöndras med en annan tredimensionell form än om ytproteinets finns på plats. Denna felaktiga tredimensionella form leder till att den aktiva delen av enzymet är blockerad och kan därmed inte klyva bort den nödvändiga biten från grannmolekyler och starta den normala autokatalytiska klyvningen av SpeB. Detta kunde upphävas genom att veckla upp den felaktiga tredimensionella formen med ett denaturerande ämne. Slutsatsen är att SpeB är beroende av det ytbundna M-proteinet för att en korrekt mognad av enzymet skall ske.

I det andra arbetet användes en metod för att reda ut vilka gener som bakterien aktiverar i olika miljöer. Tekniken kallas RNA-fingeravtryck och bygger på att man översätter RNA till komplementärt DNA med hjälp av ett s.k. omvänt transkriptas följt av förstärkning av DNA-signalen med ett antal upprepade reaktioner med ett värmestabilt DNA-polymerase (PCR). I båda reaktionerna utnyttjas en godtycklig s.k. primer (mall) som medför att man kan förstärka signalen från gener som är okända. RNA renades från streptokocker som odlats med eller utan ett socker, glukos, i odlingsmediet för att få reda på vilka gener som uppreglerades. De genererade radioaktivt märkta produkterna separerades på en polyakrylamidgel som sedan framkallades på röntgenfilm. Detta ger ett mönster av band som representerar gener som är aktiva i den aktuella miljön. Därefter isolerades och sekvensbestämde de gener som fanns efter odling i närvaro, men inte i frånvaro av glukos. Med hjälp av likhetssökning mot en databas där hela arvsmassan från en streptokock finns tillgänglig, upptäcktes att de isolerade banden representerade ett antal gener som kodar för proteiner som framförallt är inblandade i nedbrytning av socker. Dessutom uppreglerades några komponenter i ett s.k. fosfotransferassystem. Detta är både ett sockertransportsystem och ett

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signalsystem för att förändra genuttryck som svar på sockerhalten i miljön. Detta system kan vara involverat bl.a. i den nedreglering av SpeB som kan ses vid bakterietillväxt i närvaro av glukos. Sammanfattningsvis, visades att RNA-fingeravtryck är en användbar metod när man vill studera miljöreglering av tidigare okända gener hos *S. pyogenes*.

I arbete tre identifierades ett icke tidigare beskrivet endoglykosidas, EndoS, som utsöndras från streptokockerna. Endoglykosidaser är enzymer som kan bryta ner sockerstrukturer som sitter fast på mänskliga s.k. glykoproteiner. EndoS visade sig ta bort sockerstrukturen som sitter på en undergrupp av mänskliga antikroppar, nämligen IgG. Denna sockerstruktur är mycket viktig för att IgG skall fungera normalt. Bl.a. har det visats att IgG utan socker inte kan signalera till andra delar av immunförsvaret, såsom komplementsystemet och fagocyter. Dessutom är defekt socker på IgG vanligt förekommande hos patienter med autoimmuna sjukdomar såsom ledgångsreumatism och inflammatoriska tarmsjukdomar (ulcerös colit och Crohns sjukdom). Utöver fyndet att EndoS verkar på IgG, visade det sig att cysteinproteinaset, SpeB, hade förmåga att dela antikroppsmolekylen mitt itu och därmed separera kontakten mellan den del av antikroppen som binder till den främmande partikeln och den del som förmedlar signalen vidare till andra komponenter i immunförsvaret. Båda dessa upptäckter skulle kunna innebära att streptokocken förstör antikropparnas funktion och dessutom genererar IgG utan socker som skulle kunna bidra till utvecklingen av autoimmuna sjukdomar.

I den fjärde studien undersöktes om SpeB kan förstöra andra typer av antikroppar än IgG. Det visade sig att SpeB inte bara delade de andra antikropsgrupperna IgA, IgM, IgD och IgE, utan bröt ned hela, eller stora delar av molekylerna i små fragment. SpeB har alltså en bred repertoar när det gäller nedbrytning av mänskliga antikroppar. Vidare undersökte vi om EndoS hade aktivitet på IgG i sin naturliga tredimensionella form. Anledningen till detta är att närbesläktade enzymer ofta kräver att man förstör den naturliga strukturen för att de skall komma åt sockret. Det visade sig vara tvärtom när det gäller EndoS eftersom detta enzym endast bröt ned sockret när IgG var i sin naturliga form och inte när strukturen förstörts. Det innebär att EndoS sannolikt har utvecklats för att ha maximal aktivitet på IgG i dess naturliga form och gör enzymet unikt bland hittills beskrivna liknande enzymer.

Det femte arbetet beskriver vilka funktionella konsekvenser SpeB:s och EndoS aktivitet på IgG får. I blod från individer som inte är immuna mot streptokocker tillväxer bakterierna snabbt, medan de inte tillväxer i blod från

personer som har specifika antikroppar mot bakterien. Sådana s.k. opsoniserande antikroppar renframställdes från en givare. När dessa sedan tillsattes till blod från icke-immuna givare avdödades bakterierna effektivt. Detta modellsystem användes sedan för att studera SpeB:s och EndoS effekt på antikroppsmedierad avdödning av streptokocker. Opsoniserande IgG behandlades med renframställt SpeB eller EndoS. Därefter tillsattes behandlade eller obehandlade antikroppar till blod från en icke-immun givare följt av tillsats av streptokocker. När bakterietillväxten mättes visade det sig att både EndoS- och SpeB-behandlade antikroppar var lika dåliga på att avdöda bakterier som en saltlösning. Obehandlade antikroppar var däremot effektivt bakteriavdödande. Sammanfattningsvis leder SpeB:s IgG-klyvande aktivitet och EndoS spjälkning av sockerstrukturen på IgG till kraftigt försämrad antikroppsmedierad avdödning av grupp A streptokocker.

Alla fynden i denna avhandling bidrar till fördjupad förståelse av hur grupp A streptokocker på en molekylär nivå reglerar viktiga faktorer samt hur dessa interagerar med immunförsvaret hos sin mänskliga värd. Att bakterierna har utvecklat enzymatiska system som lurar immunförsvaret demonstrerar hur väl anpassad bakterien är för att överleva i värden. Dessa enzymatiska aktiviteter kan vara av betydelse både för det akuta sjukdomsförloppet samt vid utvecklingen av autoimmuna följsjukdomar.

ACKNOWLEDGEMENTS

I am indebted to all past and present colleagues at the *Department of Cell and Molecular Biology* in general, and the “old” and “new” *Section for Molecular Pathogenesis* in particular for contributing in many different ways to this thesis and for creating an pleasant and creative atmosphere.

First, I would like to thank my supervisor and good friend *Arne Olsén* for his devotion and enthusiasm during my Ph.D. studies. I have really enjoyed our mutual sharing of problems and joys both within and outside the laboratory. I know that our friendship and interest in each others well being, both scientifically and personally, will not end November 9.

The one and only *Ulla Johannesson*. My good companion in the lab. The queen of publication gels. The completing member of the lab *Olsén* troika. Without your low-key humor, support during my down periods, and excellent lab work, this thesis would never have been written. Thank you, *Ulla*.

The young boys *Magnus Rasmussen*, *Robert Janulczyk*, and *Patrik Nyberg*. You have made me believe in myself and my data when sound, and forced me to return to the lab bench when not. Thank you for being my main critics and supporters as well as my best friends in the lab. Thank you also for many fun moments outside the lab!

The old boys *Andreas Berge* and *Per Åkesson*. Thank you for leading the way and for stimulating discussions about science and things that are more important during the IDEON lunches and elsewhere.

Thanks to *Lars Björck* for scientific inspiration, collaborations and support; to *Heiko Herwald* and *Ulrich von Pawel-Rammingen* for collaborations and scientific discussions; to *Mary Jo Wick* for showing an interest in my work and for critical reading of manuscripts; to *Inga-Maria Frick* for discussions and materials; to *Anita Berglund* and *Ingbritt Gustafsson* for solving practical problems efficiently and with a smile.

I also want to thank all my good friends outside the lab, all included. A special thanks to *Stig Miller* and all his brothers.

Thanks to all my friends and colleagues in *SULF* and *SACO*, both locally and centrally.

Thanks to the *Crafoord Foundation* for donating the portable computer on which this thesis is written.

My families, *Persson*, *Collin*, and *Östberg*. Thank you for love and support.

Last, but definitely not the least, I want to thank *Eva*, to whom this book is dedicated. Thank you for being my best friend and companion in life. I am truly amazed that you have coped with all my ups and downs during this period, and that you have put up with all my periods of absence, of both physical and mental nature. If it were not for your love and patience, I would never have made this far.

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