COMPLEMENT ACTIVATION AND REGULATION IN MUCOSAL INFECTIONS

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COMPLEMENT ACTIVATION AND REGULATION IN MUCOSAL INFECTIONS

Riina Rautemaa

UNIVERSITY OF HELSINKI
HAARTMAN INSTITUTE

Department of Bacteriology and Immunology
Haartman Institute
University of Helsinki
Helsinki, Finland

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To be publicly discussed, with permission of the Medical Faculty of the University of Helsinki, in the Small Auditorium of the Haartman Institute, Helsinki on Wednesday 5th December, 2001, at 12 o'clock noon.

SUPERVISOR

Seppo Meri

MD, PhD, docent
Department of Bacteriology and Immunology
Haartman Institute
University of Helsinki, Finland

REVIEWERS

Olli Vainio

MD, PhD, docent
Department of Medical Microbiology
University of Turku, Finland

Timo Sorsa

DDS, PhD, DiplPerio, docent
Oral Pathology Research Unit
Biomedicum Helsinki, Institute of Dentistry
University of Helsinki, Finland

OPPONENT

Reinhard Würzner

MD, PhD, Assoc. Professor
Institute for Hygiene
University of Innsbruck
Austria

Be accurate when cooking toast
Never try to guess
Cook it ‘til it smokes and then
Twenty seconds less[1].

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CONTENTS

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1 PUBLICATIONS

This thesis is based on the following original publications:

I R. Rautemaa and S. Meri.
Protection of gingival epithelium against complement-mediated damage by strong expression of the membrane attack complex inhibitor protectin (CD59).
J. Dent. Res. 75:568-574, 1996.

II R. Rautemaa, G.A. Jarvis, P. Marnila and S. Meri.
Acquired resistance of *Escherichia coli* to complement lysis by incorporation of GPI-anchored protectin (CD59).
Infect. Immun. 66:1928-1933, 1998.

III R. Rautemaa, H. Rautelin, P. Puolakkainen, A. Kokkola, P. Kärkkäinen and S. Meri.
Survival of *Helicobacter pylori* from complement lysis by binding of GPI-anchored protectin (CD59).
Gastroenterology 120:470-479, 2001.

IV R. Rautemaa, T. Helander and S. Meri
Herpes simplex -virus 1 infected epithelial and neuronal cells differ in their susceptibility to complement attack.
Submitted, 2001.

2 ABBREVIATIONS

AP	Alternative pathway of complement
AdP	Adult periodontitis
BM	Basement membrane
BSA	Bovine serum albumin
C	Complement
C1INH	C1 inhibitor
CD59	Protectin
CFU	Colony forming unit
CN	Clusterin
CP	Classical pathway of complement
DAF	Decay-accelerating factor (CD55)
EDTA	Ethylene-diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
fH	Factor H
fI	Factor I
FITC	Fluorescein isothiocyanate
GCF	Gingival crevicular fluid
GPI	Glycophosphoinositol
HSV-1	Herpes simplex -virus type 1
IC	Immune complex
IF	Immunofluorescence
Ig	Immunoglobulin
LP	Lectin pathway
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAC	Membrane attack complex of complement
MASP	Mannose binding lectin associated serine protease
MBL	Mannose binding lectin
mCD59	Membrane form of protectin
MCP	Membrane cofactor protein (CD46)

NHS	Normal human serum
NHShi	Heat-inactivated normal human serum
NHSimm	Immune normal human serum
NHSni	Non-immune normal human serum
OD	Optical density
pAb	Polyclonal antibody
PBS	Phosphate buffered saline
PFU	Plaque forming unit
POX	Peroxidase
sCD59	Soluble form of protectin
SD	Standard deviation
SDS-PAGE	Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis
TP	Terminal pathway of complement
TCC	Terminal complement complex
VBS	Veronal buffered saline
VN	Vitronectin

3 ABSTRACT

The human complement (C) system is an essential part of the innate immune system and has a central role in inflammatory and immune responses. It participates in opsonization, chemotaxis, leukocyte activation and direct killing of bacteria and infected cells. The aim of the present study was to analyze how bacteria causing chronic infections and host cells at the sites of inflammation protect themselves against C attack.

In the first study C activation and regulation were investigated in adult periodontitis (AdP). AdP is a chronic inflammatory disease of the tooth supporting apparatus. Coarse granular deposits of C components C3d and C9 were seen in the subepithelial tissues of the majority of AdP (n=18) patients. In the healthy controls (n=11) C deposits were detectable only in 1 case. C deposits were also observed on the basement membranes of both pocket and oral epithelium of both healthy and AdP gingiva. In healthy gingiva CD59 was strongly expressed on the membranes of epithelial cells and on the vascular endothelia of the underlying connective tissue. In AdP CD59 was strongly expressed by endothelial cells but in the epithelia the expression was granular and weaker than in the healthy gingiva. C9 deposits were never seen at sites of CD59 expression. The gingival epithelium and connective tissue endothelia are thus well protected against damage by the membrane attack complex of C (MAC). Protection of the underlying connective tissue is, however, insufficient and may allow deposition of MAC and autologous tissue damage in AdP.

Some microbes can evade the host immune system and cause chronic infections. The second study examined whether the gram-negative bacterium *E. coli* could acquire to its outer cell membrane the glycophosphoinositol- (GPI-) anchored C inhibitor CD59 released from host cells. CD59 is a human cell membrane regulator of C that inhibits the formation of MAC. Analysis by using radiolabeled CD59,

immunofluorescence microscopy, flow cytometry and whole cell ELISA demonstrated that CD59 bound to nonencapsulated *E. coli* strains EH237 (Re) and EH234 (Ra) in a calcium-dependent manner. The incorporation required the GPI-phospholipid moiety since no binding of a phospholipid-free soluble form of CD59 was observed. Mg^{++} did not enhance the binding and a polysialic acid capsule prevented it (IH3080 strain, O18:K1:H8). Bound CD59 inhibited the C5b-9 neoantigen expression on C-treated bacteria. Protection against C lysis was observed in both a colony counting assay and a bioluminescence assay where viable EH234 bacteria expressing the luciferase gene emitted green light in the presence of the luciferine substrate.

The third study examined another gram-negative bacterium, *Helicobacter pylori*, which can cause a chronic infection that persists for years. It is sensitive to C lysis *in vitro* but it was observed that *H. pylori* acquires C-resistance by binding glycolipid-tailed inhibitors from the host. In noninfected mucosal gastric biopsy samples (n=6) CD59 was strongly expressed on the membranes of epithelial cells. In the *H. pylori*-infected epithelia (n=10) the expression of CD59 was granular and more focused to the mucus. *H. pylori* in the gastric pits were often positive for CD59 but negative for C5b-9. An opposite pattern was seen on the surface mucosa. *In vitro* analyses using ^{125}I-CD59 and bacteriolysis assays showed that CD59 bound to *H. pylori* and protected the CagA virulence factor expressing strains against C killing. In an ELISA assay the binding of CD59 correlated inversely with the appearance of the C5b-9 neoantigen. Binding of CD59 inhibits membrane attack complex assembly on *H. pylori* and may thereby contribute to their survival on the gastric mucosa. The results indicate that CD59 can incorporate into the cell membranes of nonencapsulated bacteria in a functionally active form.

In the fourth study the role of C activation and regulation in a viral infection was examined. *Herpes simplex* virus type 1 (HSV-1) infection in neurons is lifelong and generally asymptomatic. Reactivation of the latent infection results in skin blistering whereas the respective

peripheral neurons are rarely affected. Why the neuronal cells are spared while the epithelial cells are sacrificed is not well understood. During the first hours of HSV-1 infection *in vitro* the expression of the endogenous C regulators DAF and CD59 increased on both epithelial HES cells and neuronal Paju cells. By 12 hours the infected HES cells had lost their ability to control C attack. The expression of DAF and CD59 decreased and the cells became targets for MAC attack. In contrast, C regulator expression on the neuronal Paju cells did not decrease below the initial level and C5b-9 deposition was found only on 10% of the Paju cells at 12 hours. The results suggest that HSV-infected neuronal cells are better than epithelial cells in protecting themselves against C attack. This may contribute to the persistence of a latent HSV-1 infection in neuronal cells for prolonged periods.

In conclusion, these studies show that the GPI-anchored protein CD59 is a very dynamic complement inhibitor. It can become shed from host cells during infection and inflammation and become incorporated in a functionally active form into new cell membranes. When *E. coli* and *H. pylori* bound CD59 they increased their resistance to C membrane attack. Thus, some microbes are capable of recycling the released GPI-lipid anchored protein and use it for their own protection against host C attack.

4 INTRODUCTION

The human body encounters foreign material from the environment constantly. The first barriers skin and the mucosal surfaces need to protect the body against invasion by microbes, foreign antigens and toxic agents. The immune system should recognize and remove all foreign material but spare the body's own viable tissues. However, the immune system should also be able to recognize non-viable tissues as it is responsible for the clearance of tissue debris generated during trauma or during the normal cellular turn-over.

The human immune system can be divided into innate and acquired immunity. In the acquired immunity B and T lymphocytes play a major role. B lymphocytes give rise to plasma cells that produce antibodies capable of recognizing specific non-self antigens. T lymphocytes can recognize infected cells carrying foreign peptides on their surfaces. The acquired immunity develops slowly but it can very specifically recognize its target. In contrast, the innate immune system can act immediately when it gets into contact with foreign invaders. The innate immune system includes *e. g.* serum complement, pattern recognition molecules, cytokines, natural killer (NK) cells and phagocytic cells.

The human complement (C) system has a central role in inflammatory and immune responses. Complement was first identified as a heat-labile factor in serum that complemented antibodies in killing bacteria. We now know that C is an important defense system against microbial infections as it participates in opsonization, chemotaxis, leukocyte activation and direct killing of bacteria and of microbe-infected cells[2,3]. Complement serves also as an interface between the innate and acquired immune systems as it augments antibody responses and enhances immunological memory[4]. In addition, the C system is capable of discriminating between different targets without the help of antibodies. It has an important role in the clearance of immune

complexes and apoptotic cells, as well[5]. The C system is always active at a low rate and readily recognizes and attacks foreign material that is invading human tissues. Therefore, the host needs over 10 inhibitors to keep this powerful defence system under control.

Some microbes can evade the host immune system and cause chronic infections. The ability of a microbe to evade C activation is one of the features that makes it a pathogen[6]. Moreover, an ability to resist C is a key virulence feature of many bacteria[7]. Despite more than a century of parallel research on bacteria and the C system relatively little is known of the mechanisms whereby pathogenic bacteria can escape C-related opsonophagocytosis and direct killing. Compared with viruses bacteria have had less opportunities to acquire genetic material from mammalian cells. Instead, it is more likely that pathogenicity in bacteria has arisen more accidentally and on the basis of selection from natural mutants rather than by stealing or copying of genetic codes from the host. Factors providing C resistance in bacteria include external structures such as the capsule, peptidoglycan layer, lipopolysaccharide side chains, specific proteins on the outer membrane, proteins encoded by virulence plasmids and C regulatory proteins acquired from the host.

Oral and gastric mucosa are not sterile. In health, harmony exists between the "normal" flora and the immune system. The number of microbes is kept under control and their invasion to deeper tissues is prevented by mechanisms that provoke inflammation only minimally. If a pathogen invades the mucosa, an inflammatory reaction develops and the immune system aims to destroy the invader. In chronic conditions the infection persists despite the concerted activity of the immune mechanism. An underlying key question in the present thesis work was to examine how microbes can escape from the immune defense systems. On the other hand, it was also asked why the infection remains limited and what happens to host cells at the site of infection.

5 REVIEW OF THE LITERATURE

5.1 THE COMPLEMENT SYSTEM

The complement (C) system is always active at a low rate and it readily recognizes and surveys for foreign material entering human tissues. Over 10 inhibitors are needed to keep the powerful defense system under control. The biological importance of C is emphasized by the severe symptoms that C deficiencies cause. These are usually the result of an impaired activation or regulation of complement. Most plasma C components are produced in the liver and/or macrophages, and their concentrations in plasma are normally relatively stable[8]. C6 and C7 are produced by polymorphonuclear leukocytes which may release their constituents at local inflammation sites[9]. The membrane components are produced locally and act either as receptors for the C activation products or as regulators of the C cascade[8]. The main C components are shown in Table 1.

5.1.1 Complement activation

Complement can be activated via the classical, the lectin or the alternative pathway (Fig. 1). Activation of the classical pathway begins when C1q binds to specific antibodies on a target. C1q can also bind to certain bacteria independently of antibodies[10]. The lectin pathway is activated when the mannose binding lectin binds to appropriate target sugar residues found, for example, on mannose-rich serotypes of *Salmonellae*[11]. The alternative pathway is continuously turning over at a slow rate in an antibody-independent manner and will attack particles that are not specifically protected against C.

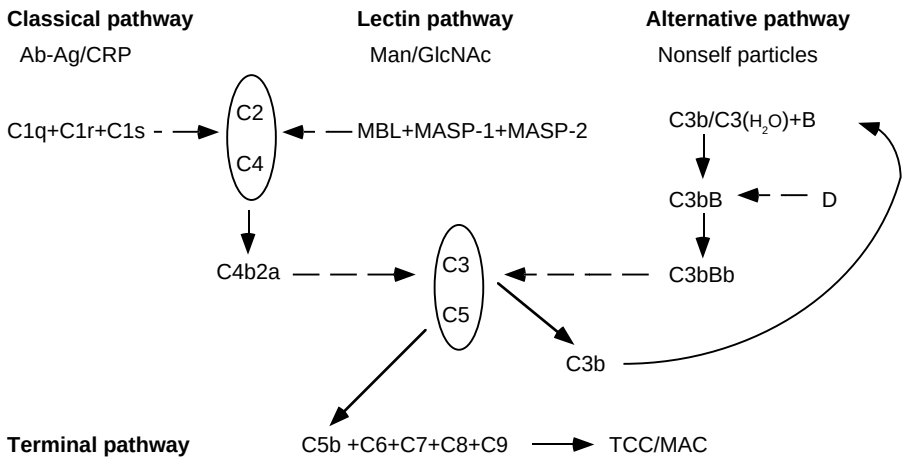

Figure 1. Activation of the complement system. The dashed lines indicate enzymatic activities. Ab-ag, antibody-antigen complexes; crp, c-reactive protein; man, mannose; glcnac, n-acetyl glucosamine; mbl, mannose binding lectin; masp, mbl-associated serine protease; tcc, terminal complement complex; mac, membrane attack complex. For other abbreviations, see text.

5.1.1.1 Classical pathway (CP)

In general, activation of the classical pathway of C begins when the first component C1 binds to antibodies bound to their antigens. The interaction takes place between the C1q portion of C1 and the Fc parts of IgG or IgM[12]. CP can also become activated by complexes containing chromatin or C-reactive protein (CRP)[13] or by surface blebs of apoptotic cells[14]. Binding to a target results in conformational changes first in C1q and then in C1r. The activated C1r-protease cleaves C1s to an active enzyme. Activated C1s is responsible for the cleavage of C4 and C2. The cleavage of C4 leads to the formation of soluble C4a and reactive C4b, which can form an amide or hydroxyl ester bond with the target surface[15]. Bound C4b forms a magnesium (Mg^{++}) dependent reversible complex with C2, which in turn becomes cleaved by C1s. The formed C4b2a complex is the C3/C5 convertase of the CP. The C2a part of the convertase cleaves C3 to C3a and C3b, and C5 to C5a and C5b. Before its

cleavage C5 binds to C3b[16]. As one convertase cleaves multiple C3 and C5 molecules the initial signal becomes enhanced.

5.1.1.2 Alternative pathway (AP)

The alternative pathway is continuously activated at a low level in plasma. C3 undergoes slow and spontaneous hydrolysis to C3(H_2O) and forms, together with Bb, the initial fluid phase C3-convertase of AP (Fig. 1). The convertase cleaves C3 and the generated metastable C3b can eventually attach covalently to amino or hydroxyl groups on target surfaces. Surface bound C3b binds factor B in a Mg^{++}-dependent manner. The B part of the complex becomes cleaved by fD and the actual C3 convertase of the AP, C3bBb, is formed[17]. The complex is stabilized by properdin. The AP can also be initiated by activation of the CP to yield the first C3b molecules. Since C3bBb cleaves several C3 molecules into C3b subunits of new C3 convertases an effective positive feedback amplification loop is generated. Amplification leads to C3b opsonization for phagocytosis, and the generation of C5b starts the activation of the terminal pathway.

5.1.1.3 Lectin pathway (LP)

The lectin pathway closely resembles the classical pathway. Instead of C1 the pathway is initiated by mannose binding lectin (MBL) that binds to carbohydrates containing mannose or N-acetyl glucosamine residues (Fig. 1). MBL belongs to a family of collectins and has structural homology to C1q. MBL binds to MBL-associated serine proteases MASP-1 and MASP-2 to form a C1-like complex. MBL binds to the target structure calcium-dependently, and the binding results in conformational changes and activation of the MASPs[18,19]. The cell-bound complex cleaves C2 and C4 similarly as C1 of the CP. The rest of the cascade is identical with that of the CP.

5.1.1.4 Terminal pathway

The terminal pathway begins when C5 becomes cleaved by either the CP or the AP C5-convertase. The formed C5b binds to C6 in the fluid phase. This induces a conformational change in C6 making it capable of binding C7. The C5b-7 complex is hydrophobic and capable of binding to lipid membranes. C8 in turn may bind either to a cell-bound or a soluble C5b-7 complex. C5b-8 binds C9 and when multiple C9 molecules polymerize and penetrate through the cell membrane, a cytolytic membrane attack complex (MAC) is formed and the membrane is perforated (Fig. 4)[20]. The terminal C complex (TCC) is a joint name for both the soluble and the membrane associated complexes (C5b-7, C5b-8, C5b-9, C5b-9_n). Clusterin and vitronectin are proteins that can keep the TCC in solution [21,22].

5.1.1.5 Anaphylatoxins C3a and C5a

Complement activation via all three pathways leads to generation of small anaphylatoxins C3a and C5a. They are involved in the generation of inflammation as they cause release of histamine, leukotrienes and other phlogistic mediators from mast cells and basophils. At low concentrations anaphylatoxins cause vascular dilatation and at higher concentrations they (particularly C5a) can cause uncontrolled contraction of smooth muscle and other characteristics of anaphylaxis[23]. The C3a receptor is expressed on eosinophils, neutrophils and monocytes and C3a has been shown to have direct immunomodulatory effects on B cells. C3a and its degradation product C3a-desArg have been demonstrated to regulate the synthesis of tumor necrosis factor-α and interleukin-β[24]. C3a and C3a-desArg also enhance cytokine synthesis in adherent monocytes while at the site of inflammation and inhibit the synthesis of proinflammatory cytokines in circulating cells. Both C3a and C5a are chemotactic and induce leukocyte migration to the site of inflammation.

Table 1. The main complement components.

Component	Pathway	Main function
C1q	CP	Binds Fc of IgG and IgM
C1r	CP	Activates C1s
C1s	CP	Cleaves C2 and C4
C4	CP, LP	C4b is a part of the CP C3/C5 convertase, C4a is an anaphylatoxin
C2	CP, LP	C2a is a part of the CP C3/C5 convertase
C3	CP, AP, LP	C3b is an opsonin and a part of CP and AP C3/C5 convertases, C3b binds fB C3a is an anaphylatoxin and chemotaxin
MBL	LP	Binds to certain carbohydrates, activates MASP-1
MASP-1	LP	Activates MASP-2
MASP-2	LP	Cleaves C2 and C4
Factor B	AP	Bb is a part of the AP C3/C5 convertase
Factor D	AP	Cleaves fB
Properdin	AP	Stabilizes the AP C3/C5 convertase
C5	TP	C5b binds C6 and C7, C5a is an anaphylatoxin and chemotaxin
C6	TP	Binds C5b and C7
C7	TP	Binds C5b6 and C8
C8	TP	Binds C5b-7 and C9
C9	TP	Binds C5b-8 and C5b-9

5.1.2 Complement regulation

If uncontrolled, activation of the complement system would lead to unnecessary consumption of C components and possible destruction of autologous cells. Therefore C activation is strictly controlled both on cell membranes and in the fluid phase by overlapping systems at various stages (Fig. 2 and Table 2).

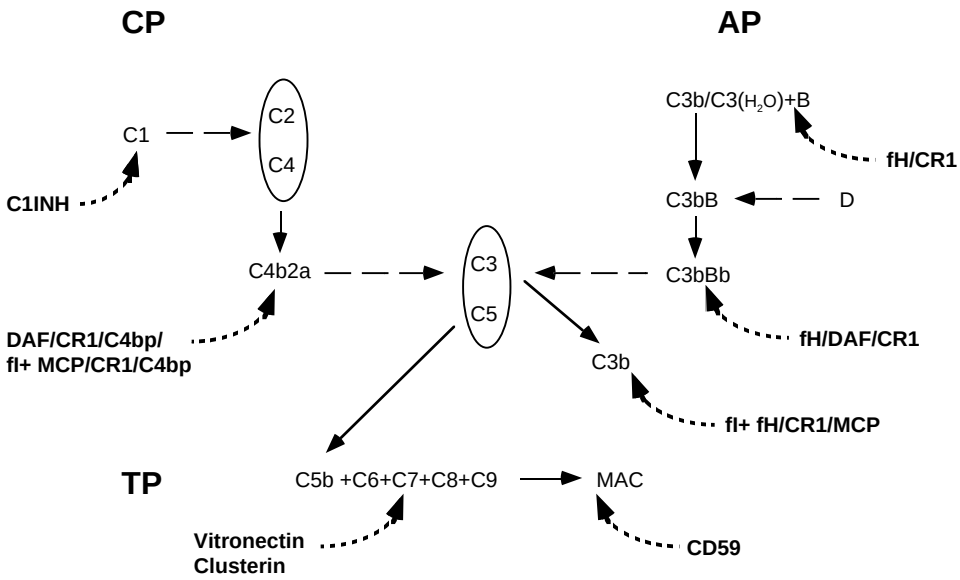

Figure 2. Regulators of the complement system. Dashed lines mark enzymatic activities, curved dashed lines mark the inhibition sites of C regulators. For abbreviations, see text.

5.1.2.1 Fluid phase regulators

Activated C1 in plasma and on immune-complexes is regulated by C1-inhibitor (C1INH; Fig. 2). Irreversible covalent binding of C1INH to activated C1r and C1s results in the dissociation of the proteins from the complex and down-regulation of the CP. C1INH is a serine protease inhibitor (serpin) and, in addition to C1r and C1s, it inhibits plasmin, kallikrein and coagulation factors XIa and XIIa[25].

Three soluble C regulatory proteins, factor H (fH), factor I (fI) and C4b binding protein (C4bp), are involved in the regulation of C3 convertases (Fig. 2). Factor H acts as an accelerator of the decay of the AP C3 convertase, as a competitor of fB for binding to C3b and as a cofactor for fI in C3b cleavage[26]. In addition, fH seems to be capable of discriminating between activating and non-activating surfaces *i.e.* to participate in the recognition of foreign particles[27]. This function is mediated by binding of fH to sialic acid and/or glycosaminoglycans on self surfaces. Factor I cleaves C3b first to iC3b and subsequently to C3c and C3dg (Fig. 3). In addition, it cleaves C4b to C4c and C4d. Factor H (AP), C4bp (CP), complement receptor 1 (CR1; AP, CP) or membrane cofactor protein (MCP; AP, CP) are used as essential, but alternative, cofactors in the cleavages (Fig. 3)[28]. C4bp also accelerates the decay of the C3/C5 convertase of the CP[29].

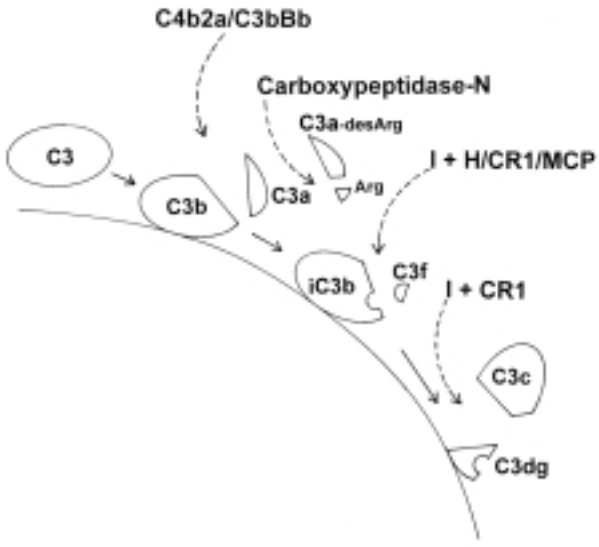

Figure 3. Activation and inactivation of C3. C3 is cleaved by the CP or AP C3 convertase into the anaphylatoxin C3a and the cell bound opsonin C3b (or free C3b). C3b is inactivated in two steps: first into iC3b by fI in the presence of a cofactor and then to C3c and C3dg by fI and CR1 as the cofactor. The anaphylatoxin C3a is inactivated by the removal of the C-terminal arginine group. The AP C3 convertase is inactivated by fH, DAF or CR1 whereas the CP C3 convertase is inactivated by C4bp or CR1. Dashed lines indicate enzymatic activities.

Vitronectin (VN) and clusterin function at the C5b-7 level. The binding of these regulators to the C5b-7 complex prevents the insertion of the complex into the membranes and thus inhibits the formation of MAC[21,22].

5.1.2.2 Membrane regulators

The membrane cofactor protein (MCP, CD46) is a glycoprotein with a transmembrane domain and an intracellular cytoplasmic tail (cyt1 or cyt2). It acts as a cofactor for fI and promotes the cleavage of C3b to iC3b or C4b to C4c and C4d (Fig. 2)[30]. It is expressed by various cells including leukocytes and endothelial cells. As an exception, red blood cells do not express MCP.

The complement receptor type 1 (CR1, C3b receptor, CD35) is a transmembrane glycoprotein. It has decay accelerating activity and cofactor activity for fI in cleaving cell-bound C3b first to iC3b and subsequently to C3dg and fluid phase C3c (Fig. 3). CR1 also has a central role in the clearance of immune complexes that have activated C as it can act as a receptor for complex-bound C3b, C4b, C1q and iC3b[31,29].

Decay accelerating factor (DAF, CD55) is a glycosyl-phosphatidyl-inositol- (GPI-) anchored membrane glycoprotein that inhibits C at the C3 level. It accelerates the decay of both CP and AP C3/C5 convertases on the cells that express it. It is expressed on most cells and the expression is upregulated by various pro-inflammatory activators of cellular second-messenger stimuli[31,29].

Protectin (CD59) is also a membrane glycoprotein linked to cell membranes via a GPI-anchor. It inhibits C at the TCC level by preventing the C5b-8 complex-catalyzed insertion and

polymerization of C9 into cell membranes (Figure 4)[32,33,34]. It is strongly expressed on most cells and in some cases the expression can be further upregulated by various pro-inflammatory activators. It is also found in most body fluids, for example in urine, milk and seminal plasma[35,36,37,38].

The GPI-anchored proteins have a tendency to become shed from cell membranes. The effective cytolysis inhibiting forms of DAF and CD59 have a glycophospholipid-tail but the phospholipid part is lost from the soluble forms[39,40,41]. Phospholipases C and D can potentially cleave off the glycophospholipid-tail and result in the formation of soluble proteins. On the other hand, phospholipase A_2 may cause a more general perturbation of the cell membrane and release host cell GPI-anchored proteins together with their anchor phospholipids. Shedding of CD59 together with its GPI-lipid moiety has been shown to allow the protein to transfer from one cell surface to another *in vitro* [42] and *in vivo* [43].

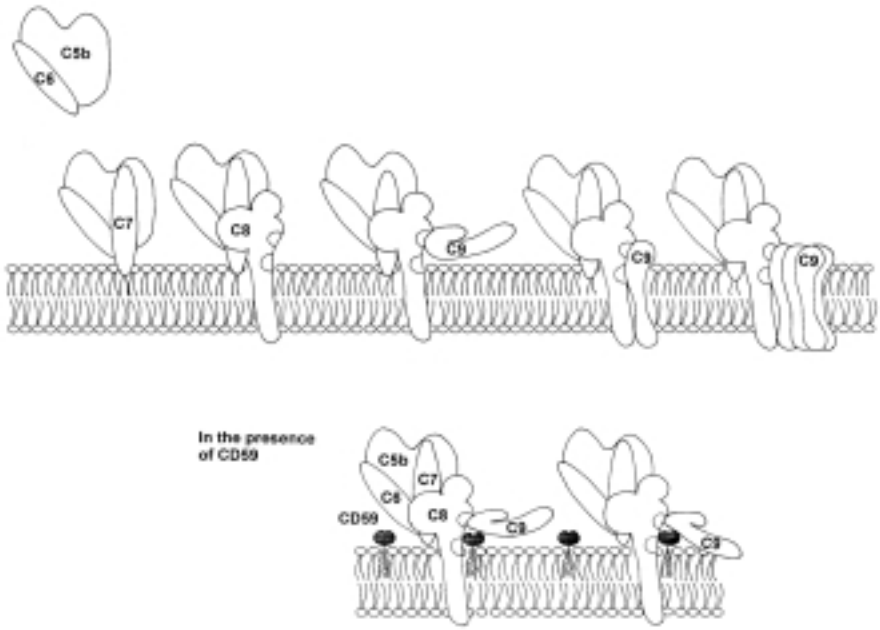

Figure 4. Top: a schematic depiction of terminal pathway component deposition on an eukaryotic cell membrane. Bottom: terminal pathway and the function of CD59 (filled). CD59 inhibits the insertion and polymerization of C9 of the MAC.

Table 2. The main complement regulators.

Protein	Recognition	Function
Soluble regulators		
C1 inhibitor	C1r, Cs	Dissociation of C1s and C1r from the C1 complex after covalent binding
Factor I	C3b, C4b	Degradation of C3b and C4b
Factor H	C3b	Decay accelerating activity for the C3/C5 convertase of AP, cofactor for fI
C4b binding protein	C4b	Decay accelerating activity for the C3/C5 convertase of CP, cofactor for fI
Clusterin	C5b-7	Prevention of C5b-7 membrane insertion
Vitronectin	C5b-7	Prevention of C5b-7 membrane insertion
Membrane regulators		
CR1 (CD35)	C3b, iC3b, C4b	Decay accelerating activity for the C3/C5 convertases, cofactor for fI, immune complex transport
MCP (CD46)	C3b, C4b	Cofactor for fI
DAF (CD55)	C3b, C4b	Decay accelerating activity for the C3/C5 convertases
Protectin (CD59)	C8, C9	Inhibition of MAC

5.2 COMPLEMENT AND INFECTIONS

5.2.1 General

The clinical outcome of all infections is strongly modulated by the complement system as it participates both in the neutralization of invading microbes and in the generation of inflammation. Complement-derived anaphylatoxins cause increased vascular permeability, and chemotaxins attract leukocytes to the site of microbial invasion. Both gram-negative and -positive bacteria can become opsonized with C3b and iC3b, and the opsonized bacteria become phagocytosed. MAC can be formed on gram-negative bacteria exposing them to the lytic action of C. Although the formation of MAC has been shown to be necessary for optimal killing of some gram-negative bacteria[44,45], opsonophagocytosis remains the major antibacterial defense mechanism. Virus-infected cells and virions may also become opsonized with C3b, iC3b or specific anti-viral antibodies capable of activating the CP[46].

5.2.2 Complement deficiencies

The complement system consists of more than 30 proteins, many of which have multiple functions. Different deficiencies therefore result in different clinical consequences. The major disorders caused by C component deficiencies are recurrent infections and immune complex (IC) diseases[47]. The deficiencies of C regulators can also result in diseases with severe symptoms.

Three types of C component deficiency have been reported to explain an increased susceptibility to pyogenic infections. Any deficiency that affects opsonization causes a general susceptibility to pyogenic microbes whereas defects in the lytic action of C increase the susceptibility to neisserial infections[48]. Defense against

disseminated neisserial infection is dependent on the recognition of the bacterial surface by antibody and complement. Individuals with inherited deficiences in the terminal C components, which are critical for neisserial killing, often experience disseminated systemic or relapsing neisserial infections despite having an intact opsonophagocytic system. The fact that these patients are not more prone to other systemic infections, caused by *e.g. Haemophilus influenzae,* suggests that there is a unique relationship between the neisserial cell surface and the C system. Deficiencies of the lectin pathway have been reported to result in an increased risk for recurrent respiratory infections in early childhood[11].

C1q deficiency causes a failure of the classical pathway and accumulation of immune complexes (IC)[49]. C1q deficiency has been related to the pathogenesis of systemic lupus erythematosus (SLE) because it is a disease where ICs accumulate in different organs. C1q is involved in the clearance of ICs and the balance between C1q and IC is lost in SLE. C1q also participates in the clearance of apoptotic cells. Impaired clearance of these cells and their fragments has been found to predispose to autoimmune conditions[50]. C1q deficiency has also been associated with a susceptibility to infections caused by encapsulated bacteria and fungi[47].

C1INH deficiency results in a loss of regulation of C1 and a failure to activate kallikrein. Hereditary angioedema (HAE) is caused by a reduced C1INH level (type I) or by a reduced C1INH functional activity (type II). Low C1INH leads to reduced levels of C2 and C4. HAE is an autosomal dominantly inherited disease or occurs as a sporadic “de novo” mutation in the C1INH gene. The patients have characteristic episodes of painless swelling of the skin and mucosal membranes[4].

Dysfunction of factor H can cause two types of kidney diseases, either membranoproliferative glomerulonephritis type II (MPGN II) or hemolytic uremic syndrome (HUS). MPGN II is the consequence of a

total lack of factor H (or its function) whereas HUS develops when mutations in the fH gene affect the function of the most C-terminal domains of fH[51,52,53].

Paroxysmal nocturnal hemoglobinuria (PNH) is a hemolytic disease characterized by an increased sensitivity of a proportion of erythrocytes to C-mediated lysis, granulocytopenia, thrombocytopenia and a tendency for venous thrombosis[54]. The increased susceptibility of the cells to C is due to their lack of DAF and CD59. PNH is usually caused by a clonal somatic mutation in the PIG-A gene that affects the GPI-anchor synthesis[55]. Isolated deficiencies of the other membrane regulators are rare.

5.2.3 Adverse effects of C activation

The primary function of the C system is to protect the host against microbial invasion. C also participates in the processing of non-self material and in the removal of debris of self-origin. In most cases, the C system is well controlled and inappropriate activation and host cell destruction are prevented. However, C activation is not always entirely beneficial to the host. During bacteremia a vast amount of anaphylatoxins C3a and C5a are formed. They may lead to a violent enhancement of inflammation and septic shock. The C system becomes activated at a site of tissue necrosis and the clearance is not totally restricted to the necrotic site itself. For example, in myocardial infarction C activation is an inducer of the reperfusion injury[56,57]. The inflammatory changes during cardiopulmonary bypass are also partially caused by activation of the C system and the terminal pathway[58,59]. Autoantibodies present in autoimmune diseases activate the CP, which can result in an enhancement of inflammation and C attack against autologous cells.

5.2.4 Adult periodontitis

_Adult periodontitis (AdP) is a chronic inflammatory disease of the tooth supporting apparatus. It is characterized by loss of clinical tooth attachment due to destruction of the periodontal ligament and loss of the adjacent supporting bone[60]. An important feature of AdP is the accumulation of gingival crevicular fluid (GCF) in the gingival crevice of periodontal lesions (Fig. 5). A healthy gingival crevice produces small amounts of GCF, but production is markedly increased at a site of inflammation. Inflammatory cells, neutrophil granule constituents and a variety of plasma proteins including IgG, IgM, IgA and interleukins have been identified in this fluid[61,62,63].

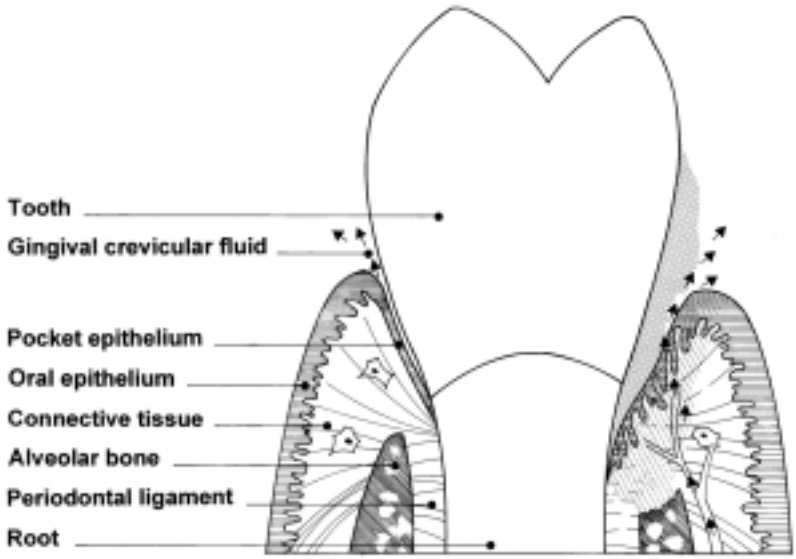

Figure 5. A schematic structure of healthy (left) and chronically infected AdP (right) periodontium.

AdP results from a complex interplay of a mixed bacterial infection and host response. In addition, behavioral factors, like cigarette smoking, modify the outcome of the disease. There is no direct pathogen-disease link in human periodontitis but the presence of some bacteria has a strong association with progressive disease[64]. It has also been suggested that Herpes viruses have a role in the

development of destructive periodontal disease[65]. In AdP proteolytic interstitial collagenases (matrix metalloproteinases) have a central role in the periodontal degradation process[66]. The progression of the disease can be stopped by inhibiting or down-regulating the activity of the matrix metalloproteinases -8 and -13[67]. These enzymes have been shown to originate predominantly from neutrophils[68], which are recruited to the site of inflammation *e.g.* by the chemotactic C fragments (Fig 6).

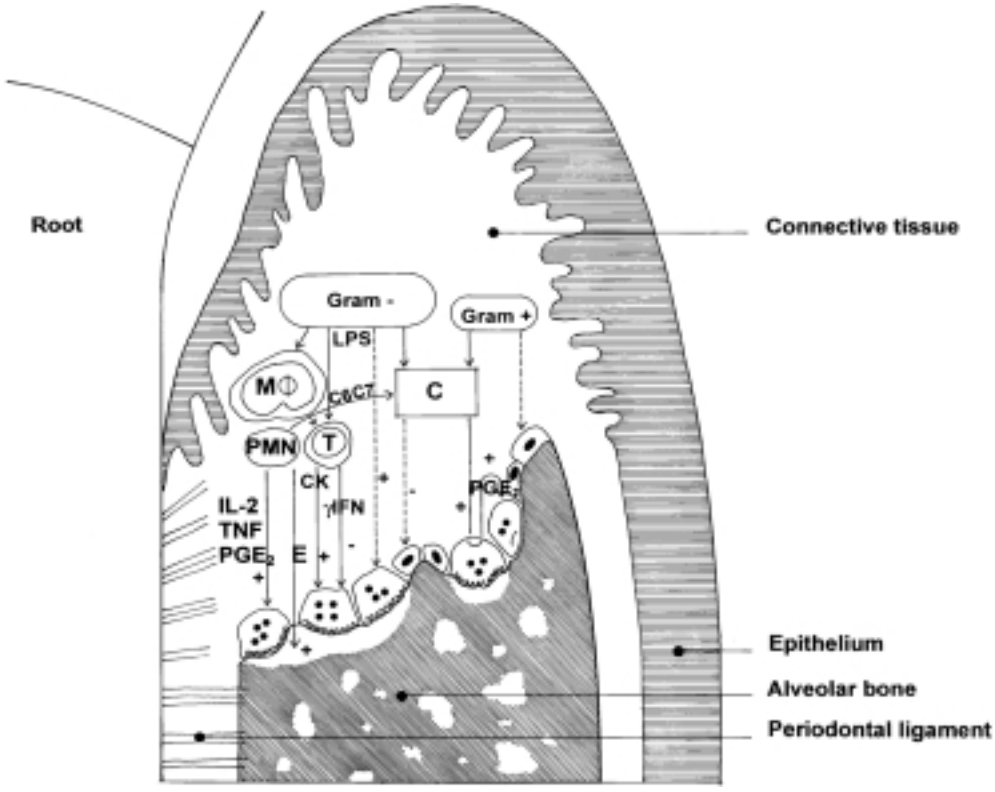

Figure 6. An enlargement of the chronically infected area of AdP periodontium. Mechanisms active in periodontal bone resorption: Mϕ, macrophage; PMN, polymorphonuclear neutrophil; T, T-lymphocyte; C, activated complement system; E, enzymes causing breakdown of the organic bone matrix; IL-1, interleukin-1; TNF, tumor necrosis factor; PGE_2, prostaglandin E_2; CK, cytokines; γ-IFN, γ–interferon; LPS, lipopolysaccharide of gram-negative bacteria. Activated osteoclasts are marked with multiple dots, resting osteoclasts with single dots.

One of the strongest associations between a suspected pathogen and destructive periodontal disease is provided by *Actinobacillus actinomycetemcomitans* (A.a.). Although A.a. has especially been implicated in juvenile forms of periodontitis, it has also been associated with AdP[69]. *A. actinomycetemcomitans* is a small, nonmotile, gram-negative, aerobic rod which causes a strong antibody response especially in juvenile periodontitis. *Porphyromonas gingivalis* is another probable periodontal pathogen associated with the development of AdP [70]. It is a gram-negative, anaerobic, nonmotile, short rod of the black-pigmented *Bacteroides* group, the members of which produce an exceptionally large array of virulence factors like many proteases, endotoxin, NH_3 and H_2S[71]. In response to an infection by *Bacteroides*-bacteria a local and systemic immune response develops.

Activation products of the C components have been detected in the GCF at the site of inflammation[72,73]. C3d, an activation product of C3, is known to be present at high levels in the GCF of AdP patients, but not in serum, suggesting a local activation of the C system[74,75]. It has also been observed that the proportion of native C3 in GCF significantly increases after periodontal treatment[76]. The rare occurence of C4 cleavage in periodontal diseases suggests that C activation in AdP occurs mainly *via* the alternative pathway[74]. As C activation poses a threat to the nearby host cells, these should be protected against C-mediated damage.

5.2.5 *H. pylori* gastritis

Helicobacter pylori is the causative agent of chronic gastritis and the single most important factor in peptic ulcer disease[77]. There is also a strong association between *H. pylori* infection and gastric cancer[78]. However, it is not known why only a minority of *H. pylori*-infected patients develop severe sequealae, nor are the pathogenetic mechanisms underlying *H. pylori* infection well understood. It has been suggested that the differences in the outcome of the disease are due to various virulence factors present in different *H. pylori* strains. The flagella and specific adhesins seem to be important in *H. pylori* colonization whereas a vacuolating cytotoxin and lipopolysaccharide mainly influence the degree of inflammation in the gastric mucosa. *H. pylori* urease seems to be necessary for controlling the acidity during initial colonization [79]. The presence of the cytotoxin-associated gene A (*cagA*) locus has been shown to be associated with the peptic ulcer disease and atrophic gastritis. It is the best known virulence factor of *H. pylori* although the actual function of the CagA protein is still not known.

H. pylori induces a strong inflammatory response in the gastric mucosa and expression of a wide spectrum of cytokines. Neutrophils accumulate in the mucosa together with macrophages, lymphocytes and plasma cells[80]. Both local and circulating antibodies can regularly be demonstrated in infected patients[81]. All the machinery needed for immune defense thus seems to be present, but still a spontaneous recovery is rare and in the absence of treatment chronic *H. pylori* infection persists for years. *H. pylori* bacteria are mainly found in the mucus of the gastric lumen and only rarely within tissues. How *H. pylori* survives in the gastric mucus is still incompletely understood.

To colonize the human stomach *H. pylori* must survive the acidity of the gastric juice, traverse through the gastric mucus layer and evade the human C system, phagocytosis and other defense

mechanisms. *H. pylori* tolerates the acidity by producing large amounts of urease that can hydrolyze gastric juice urea into ammonium[82]. *H. pylori* urease also reduces opsonization by C and thus interferes with the phagocytosis by granulocytes[83]. So far, no answer has been offered to explain how the bacterium evades the lytic action of complement present in the gastric mucus and mucosa.

Helicobacter pylori bacteria do not survive in human serum because of their susceptibility to the cytolytic activity of the plasma C system[84,85]. Furthermore, it has been shown that unless opsonized by the alternative pathway of C *H. pylori* may survive in phagocytes[86]. The C system has been shown to become activated in *H. pylori*-positive and negative gastritis[87]. C3b, as well as soluble terminal C complexes (TCC), have been found in the gastric mucus.

5.2.6 Herpes simplex type 1 infection

Herpes simplex -virus type 1 (HSV-1) infects the majority of the human population. Although the primary infection is usually efficiently controlled by the immune system, the infection is lifelong and complete clearance of the virus is seldom achieved. Latent virus conceals itself in trigeminal, vagal or sacral ganglia and viral gene expression is minimal during latency. The infection becomes periodically reactivated resulting in virus shedding and recurrence of symptoms. In addition to the classical symptoms of labial and mucosal blistering, recurrent infections have also been linked to post-herpetic neuralgia and facial paralyses. Severe manifestations, like encephalitis, occur mainly in the newborn or in immunocompromised individuals.

Both humoral and cellular defense mechanisms contribute to resistance against HSV-1 infection and its disease manifestations[88]. HSV-1 has developed many strategies to survive in the human host[89]. One of the most important ones is its ability to establish a latent

infection in neurons. During the latent state viral genes are not expressed and the exposure to the immune system is minimal. To be able to infect another host the virus must, however, reactivate and migrate from the neuronal cells. Reactivation and virus replication is secured by the production of specific immune evasion molecules capable of blocking antibody or C activity and preventing antigen presentation by the HLA class I complex.

Herpes-viruses have been shown to encode proteins which interact with human C proteins. Some of these are thought to prevent C activation and others mediate virus entry into host cells (e.g. gp330 of the Epstein-Barr-virus). HSV-1 glycoprotein C (gC-1) is a relatively well characterized viral immune evasion protein[90,91]. It binds to C3, C3b, iC3b and C3c and inhibits alternative pathway C activation. In addition to protecting the cell-free virus from C-mediated neutralization the viral C regulators play a role in limiting host cell destruction during the infection. In the absence of gC, HSV-1 is readily neutralized by C by a C5-dependent mechanism[92]. Thus, humoral defense against HSV is probably more dependent on antibodies and the classical pathway of C than on the alternative pathway. Actually, inherited partial C4 deficiencies predispose to frequent and unusually severe intraoral HSV-1 infections[93]. While the virus can effectively inhibit the alternative C pathway the host is dependent on the classical pathway for defending itself against severe infections.

Since HSV-1 has minimized its exposure to the immune system and controls immune attacks it has been able to develop a long lasting relationship with its host. It has been equally important for the host to learn to prevent unnecessarily strong immune responses and autologous cell destruction[94]. This is especially important when the infected tissue is vital and poorly regenerating.

5.3 COMPLEMENT AND MICROBES

5.3.1 Complement resistance mechanisms of bacteria

5.3.1.1 General

As the complement system is a powerful defense system of the host, any pathogenic microbe coming into contact with human blood or plasma must have developed mechanisms to evade C attack. Most gram-negative bacteria are sensitive to the lytic action of C in fresh human serum, whereas some are resistant and therefore more virulent. Some completely serum resistant strains of gram-negative bacteria can actually grow in serum. The spectrum of the C resistance mechanisms of microbes is far wider than our knowledge of it. Interactions of each bacterial species with different components of the large group of C components are involved in the generation of distinct syndromes that each of the bacteria can cause. We are still at a very early stage in understanding the C evasion mechanisms of bacteria. Exploration of genomes of bacteria will provide the basis of search for virulence factors that interfere with the C system. This task is not as simple as in the case of viruses, where readouts of the genomes have directly pointed out proteins that are structurally homologous to human C inhibitors.

5.3.1.2 LPS

Lipopolysaccharides (LPS) are a major constituent of the outer membranes of gram-negative bacteria. LPS becomes released from bacterial surfaces at cell death and also from the surfaces of intact bacteria in smaller amounts. LPS is also called endotoxin since it is responsible for many noxious effects during an infection. LPS can activate the classical and the alternative C pathways and in some

cases (*e.g. Salmonella* O:6,7 LPS) also the lectin pathway. LPS can cause strong inflammatory reactions also independently of C. The *O*-polysaccharide side chains of LPS can sterically hinder the access of C components to the bacterial membrane. Long *O*-polysaccharide side chains of LPS can prevent C1q from binding to the lipid A part of LPS and activating the classical pathway. Most *E. coli* and *Salmonella* strains readily bind and activate C1, but C1INH stops the activation at an early stage on smooth strains containing long *O*-polysaccharide side chains[95]. Long-chain LPS appears to be the major determinant of serum resistance of *Salmonellae*[96]. In this case C resistance depends both on the quantity and structure of the *O*-antigen side chains[97,98]. Strains with smooth LPS show less bound C3b than their rough, *O*-side chain deficient counterparts after treatment with serum.

Activation of the alternative C pathway on target cell surfaces is regulated by an interaction between surface-bound C3b and the key regulator factor H. Factor H inhibits the alternative pathway C3 convertase and promotes inactivation of C3b into iC3b by factor I (Fig. 2). Negatively charged sialic acid can enhance the binding of factor H to C3b on the cell surface and prevent amplification of the alternative pathway[99,100,101]. Since most microbes lack sialic acid on their surfaces they are “activators” of the alternative pathway. However, the presence of sialic acid on the bacterial surface can provide resistance against C attack. This has been suggested to be due to a direct interaction of the cell surface sialic acid with factor H[101]. Meningococci can have a sialic acid capsule (group B) but sialic acid also occurs as a terminal sugar on the lipo-oligosaccharide (LOS) of both meningococci and gonococci[102]. Serum resistant strains of gonococci are known to be more sialylated than the serum sensitive ones. This leads to lower amounts of C3b and iC3b on the bacterial surface[103]. Gonococci can also vary the sialylation of their LOS structures during infection. Low level sialylation is important for entering mucosal epithelial cells but high level sialylation gives protection against C lysis[104]. Ram et al[105] have recently located a gonococcal LOS sialic acid binding site on factor H. The binding of

SCRs 16-20 of factor H to gonococcal sialic acid blocked C activation at the level of C3. *Neisseriae* can sialylate their LOS glycolipids either endogenously or exogenously but it has been suggested that exogenously sialylated gonococcal LOS can interact with factor H whereas endogenously sialylated meningococcal LOS and meningococcal sialic acid capsule cannot[103]. A study by Estabrook et al[106] showed that the serum sensitivity of group C meningococci correlated with the expression of free sialic acid acceptor sites on their LOS. Both endogenous and exogenous LOS sialylation were associated with an increased serum resistance by masking free acceptor sites.

In addition to preventing C attack at the C3 level the LPS side chains can restrict MAC assembly on the microbial surface. Opsonization with C3b and iC3b is important for the intracellular pathogen *Burkholderia pseudomallei* to gain entry to human phagocytic cells and establish infection. Although C is activated on the surface of *B. pseudomallei* the cells are not lysed by MAC. This has been suggested to result from a weak ionic binding of C5b-9 to the polysaccharide side chains of LPS instead of the bacterial membrane[107]. It has also been shown that a long *O*-polysaccharide side chain of *Salmonellae* can prevent insertion of the forming MAC into the outer cell membrane and lead to its shedding[96].

5.3.1.3 Capsule and peptidoglycan

The bacterial capsule has a very important role in protecting microbes against the C attack they eventually encounter in the human body. Polysaccharide capsules have been shown to inhibit phagocytosis of both gram-positive and gram-negative bacteria. The capsules and the thick peptidoglycan layer of gram-positive bacteria sterically hinder the access of C molecules to the bacterial surface. Even if the opsonins C3b or iC3b were formed they may become embedded deep in the capsular network and be inaccessible to their receptors on phagocytes. This has been most clearly demonstrated

with *Staphylococcus aureus*[108], virulent pneumococci[109] and *Escherichia coli*[110]. However, opsonization with C is essential for the phagocytic killing of many encapsulated bacteria.

The capsule is a major C resistance and virulence factor for a number of pathogens. For example, capsule-deficient strains of *Staphylococcus aureus* are opsonized for phagocytosis by C alone whereas strains with a proper capsule need both specific antibodies and C for their phagocytosis[111]. Also, nearly all invasive strains of *Haemophilus influenzae* (Hib) are encapsulated. Patients with invasive Hib disease have often multiple copies of the genes responsible for the capsule expression[112] and it has been shown that amplification of these genes increases the resistance of the bacteria to complement-dependent host defense[113]. Capsules of serogroup B disease-causing meningococci are rich in sialic acid which is relatively nonimmunogenic in human hosts and prevents C attack[114]. Group B streptococci with type III capsular polysaccharides containing sialic acid residues have been shown to inhibit C3 deposition by inhibiting the AP[115]. The exact mechanism for this has not been defined yet, but as one possibility it has been thought that sialylated structures bind factor H, the main fluid phase regulator of the AP C3 convertase. On the other hand, colominic acid, an essential component of the sialic-acid capsule of *E. coli* K1, which is structurally identical to the serogroup B meningococcal capsule, does not seem to interact directly with factor H[101]. The conflicting results of the ability of sialic acid in capsules and lipo-oligosaccharides to bind factor H probably results from the fact that in the former case sialic acids are in a polymeric configuration and in the latter case as terminal sugar moieties. Differences in the organization of the capsular sialic acid polymers may also cause differences in the amount of accessible terminal sugar moieties and binding sites for factor H.

The steric barrier of the capsule and peptidoglycan also hinder very efficiently the access of the lytic membrane attack complex (MAC) to the membrane. Mainly due to their thick cell wall gram-positive

bacteria are not lysed by C. Accordingly, many of the encapsulated gram-negative bacteria can evade the lytic action of C although there are other more specific means for C resistance for these bacteria. Ward and Inzana[116] have reported that capsular polysaccharides of *Actinobacillus pleuropneumoniae* can limit the amount of bound C9 and the sensitivity of these bacteria to C lysis although they do not limit the early C activation. The role of the capsule of gram-negative bacteria in C resistance has been given much less attention than that of gram-positive bacteria.

It has been thought that C-reactive protein (CRP), an acute phase reactant, participates in defense against pneumococci by binding to the pneumococcal cell wall C polysaccharide (CPS) and activating the classical pathway. However, CPS is beneath the capsule and not accessible to CRP except in aged bacteria. It has also been observed that CRP can inhibit the alternative pathway of C[117]. There is also the possibility that pneumococci may utilize CRP for the evasion of the alternative pathway. Microbes that bind CRP could in principle exploit it for their own benefit to avoid alternative pathway attack. It has been observed that CRP binds the key alternative pathway inhibitor, factor H, which could give the microbes protection against alternative pathway activation[118,119]. Plasma levels of CRP rise up to 100-1000-fold during invasive bacterial infections and the physiological purpose of this is still somewhat obscure. Since CRP has been observed to activate the classical pathway and inhibit the alternative pathway it is possible that it participates in the clearance of cell remnants and other material via the activation of the classical pathway and suppresses excessive alternative pathway-mediated inflammatory responses at locations where it has become bound. By increasing binding of factor H to surfaces coated with C3b CRP may also promote inactivation of C3b into iC3b and generate ligands for phagocytosis by macrophages[119].

5.3.1.4 Membrane proteins

A classical example of a specific factor responsible for C resistance of a microbe is the M protein family of *Streptococcus pyogenes.* M proteins show great structural and antigenic variation between strains and are considered to be the major virulence factor of *S. pyogenes*. All molecules in the M protein family are fibrillar coiled-coil dimers with variable N-terminal sequences. Most M proteins bind human fibrinogen and sterically hinder the binding of C molecules to the streptococcal cell wall[120,121]. Some M proteins inhibit phagocytosis and allow the bacteria to grow in whole human blood but the functions of many of them are still unknown[122]. M protein can selectively bind factor H and thereby effectively block the activity of the alternative pathway C3 convertase[123]. Recently, the M protein binding site on factor H was mapped to the SCR7 domain[124]. This site binds also heparin but does not interact with C3b. Therefore, binding of factor H by M protein does not compromise the factor H-mediated C inhibitory activity. It has recently been shown that the serum resistant strains of Lyme disease pathogen *Borrelia burgdoferi* have surface proteins capable of binding fH and fH-like protein 1[125]. This interaction promotes fI-mediated degradation of C3b on the bacteria and their evasion from C attack[126,127].

It has also been shown that the highly variable region of many members of the M protein family, *e.g.* Emm, can bind C4bp in a functionally active form[128,129]. C4bp regulates the classical pathway by decaying the classical pathway C3 convertase and by acting as a cofactor for factor I in degrading C4b. The gram-negative bacterium *Bordetella pertussis* expresses on its surface a filamentous hemagglutinin which has for long been related to the virulence of *B. pertussis.* Filamentous hemagglutinin has recently been shown to be the C4bp binding structure on *B. pertussis*[130].

Porins are the major outer membrane proteins of gonococci and occur in two primary immunochemical classes Por1A and Por1B[131]. Strains expressing Por1A have been associated with serum resistance and disseminated disease. It was recently shown by Ram and colleagues that Por1A can specifically bind factor H independently of lipo-oligosaccharide (LOS) and thus downregulate the alternative pathway of C[132].

A factor on *Y. enterocolitica* associated with resistance to C is the outer membrane protein Ail. When expressed in *E. coli* Ail has been shown to inhibit C at the level of C5b-9[133]. Rck is an outer membrane protein of *Salmonella typhimurium,* structurally as well as functionally homologous to Ail[134]. The C resistance mediated by Rck is associated with a failure to form fully polymerized tubular MAC, a mechanism analogous to the function of human CD59 (CD59)[135]. The TraT lipoprotein of *Salmonellae* and *E. coli* increases the resistance of the bacteria to C killing probably by interfering with the formation of C5b6 and correct assembly and membrane insertion of the MAC[136].

Many bacteria have proteases that can inactivate C proteins or inhibit their accumulation on bacterial surfaces[137,138]. For example, proteases expressed by *Porphyromonas gingivalis* have been shown to be major virulence factors for the microbe. *P. gingivalis* expresses a wide spectrum of proteases and some strains express an arginine-specific cysteine protease capable of cleaving C3 and C5[139]. C3 is cleaved into C3a-like and C3b-like molecules but C3b does not become bound onto the bacterial surface[140]. The C5a-like biologically active chemotactic factor cleaved from C5 can recruit neutrophils to the site of infection and thus result in progression of the disease[141]. Many strains of group A and B streptococci produce a C5a inactivating C5a-ase and can therefore inhibit the inflammatory response and opsonophagocytosis[142]. Streptococci and staphylococci can specifically bind and activate plasminogen which provides the bacteria with the ability to recruit proteolytic activity from the

host[143]. Such activity could also lead to the inactivation of C3b and cessation of amplification of the C cascade[144].

5.3.1.5 Antibody blocking and antigenic variation

A surface-bound antibody normally directs and facilitates C activation to the target surface and will lead to cell lysis if C activation is not inhibited. In some instances, however, an antibody can block the lytic action of C or the binding of a specific C activating antibody to the surface resulting in increased C resistance of the microbe. For example, binding of human IgA1 may either block or initiate C activation on meningococcal surfaces depending on the molecular site to which IgA1 binds. If IgA1 binds to group C polysaccharide capsule, it blocks lysis initiated by cocirculating IgG and IgM[145]. Blocking IgG against the gonococcal outer membrane protein Rmp prevents further activation of C in a different manner. Once bound, blocking IgG refocuses C deposition to a new site that does not lead to cell lysis[146]. Human natural anti-Gal antibody has been proposed to block the alternative pathway and cell lysis when bound to the LPS of *S. marcescens*. The mechanism for this is not known[147]. An LPS-specific blocking antibody against *A. pleuropneumoniae* has also been described[116].

Most bacteria can vary their outer membrane protein composition to some extent. Bacteria capable of varying their surface antigens can evade complement-mediated killing and opsonophagocytosis as the new antigens are not recognized by existing antibodies and the classical pathway of C does not become activated. In bacteria, however, the antigenic variation is not as wide-spread and extensive as *e.g.* on certain protozoan parasites, like trypanosomes[148].

5.3.1.6 Utilization of membrane receptors and regulators

Bacteria have various ways to exploit C proteins and C regulators for adhesion, invasion and for their own protection. Mycobacteria are intracellular pathogens that can invade host macrophages by activating the alternative pathway and thus bind to the C receptors CR1, CR3, and CR4[149]. It has also been shown that by binding C2a from the host pathogenic mycobacteria can form a C3 convertase of the classical pathway on their surfaces and utilize that during their invasion[150]. Some organisms, particularly viruses, use membrane regulators of C as their receptors when entering the human body. Of bacteria, it has been shown that some pathogenic *E. coli* strains use decay accelerating factor (DAF) on the urinary tract epithelial cells as an attachment ligand[151,152]. The ligand on the bacterial side is the Dr-adhesin.

Since the human C inhibitors DAF and CD59 have GPI-anchors they can potentially insert themselves into membranes of other cells or particles. It has been observed that DAF can be incorporated into the membrane of red blood cells[39] and CD59 can bind to high density lipoprotein (HDL) particles[42]. Lipoteichoic acids (LTA) are membrane teichoic acids of gram-positive bacteria. They become readily shed from the bacterial surface and can spontaneously bind to mammalian cell surfaces[153]. LTA bound to mammalian cells sensitizes them to autologous C and redirects C activation away from the bacterial surface at the same time[154].

5.3.2 Complement resistance mechanisms of viruses

Viruses can exist in two forms: as extracellular virion particles and as intracellular genomes. Virions are more resistant to physical stress than plain genomes but more susceptible to immune control. Virus genomes may be concealed in host cells with limited gene expression and minimal exposure to the immune system. However, to infect a new host the virus must replicate and migrate away from the old host cell. Viruses have thus evolved different strategies to evade host immune control mechanisms. These strategies include the utilization and mimicry of the C system.

5.3.2.1 C component mediated adhesion

Virions need to adhere to the host cell in order to infect it. Many viruses use C proteins for their adhesion. Epstein-Barr-virus gp350 binds to CR2 (C3d receptor; CD21) mediating virus adhesion and entry into the host cells[155]. The measles virus uses MCP (CD46) as its receptor[156] and the West Nile virus uses CR3 (iC3b receptor; CD11b/18)[157] to enter the host cell. HIV-1 gp41 mimicks C3 and is therefore capable of binding to C receptors CR 1 (C3b/C4b receptor; CD35), 2 and 3[158]. It has also been suggested that HIV-1 gp41 also binds to CR3-like molecules on *Candida albicans*[159]. This binding causes enhancement of candidal proteinase release and suppression of PMN phagocytosis. HIV-1 gp41 can thus indirectly contribute to the virulence of HIV-1.

5.3.2.2 Misdirecting the C system

Virus replication results in viral antigen production on the host cell surface and its exposure to the immune system. Some viruses can cause shedding of strongly C activating surface structures into the environment. This can lead to activation and consumption of C components in the fluid phase instead of on the host cell membrane. Upon budding several viruses coat themselves with a lipid bilayer from the host cell membrane. In addition to viral proteins, this membrane carries many "self" proteins including C regulators and may thus be recognized as a non-activator of the AP.

5.3.2.3 Mimicry of C regulators

During their evolution many viruses have incorporated gene segments of host C regulatory proteins in their genomes. The infected cells and virions can thus express functional proteins that control the C activation. For example, a vaccinia virus C control protein (VCP) has been identified and the gene encoding the protein has homology to a group of MCP-like eukaryotic C regulatory proteins[160]. Herpesvirus saimiri expresses a MAC inhibiting protein with sequence homology to human CD59[161]. It also has a structurally and functionally DAF -like protein that accelerates the decay of the AP C3 convertase. Epstein-Barr-virus expresses a protein that binds to C3b, iC3b and C4b. HSV-1 and HSV-2 have a functionally CR1-like protein (gC), whose structure, however, is distinct from CR1. The HSV-1 glycoprotein C (gC-1) binds to C3, C3b, iC3b and C3c and inhibits alternative C pathway activation[92]. HHV-8, the Kaposi's sarcoma virus, expresses a functionally C4bp/DAF-like protein that accelerates the decay of the AP C3 convertase[162].

5.3.2.4 Acquisition of host cell membrane regulators

Several viruses coat themselves with a lipid bilayer from the host cell membrane upon virion budding. The membrane carries many host cell C regulators. DAF and CD59 have been shown to be particularly important in protecting *e.g.* HIV virions from C lysis[163,164,165]. Vaccinia virus acquires DAF, CD59 and MCP upon budding. Cytomegalovirus even induces the expression of C regulatory proteins on the host cell during viral replication to assure the replication and a sufficient number of C regulators on the budding virions[166].

6 AIMS OF THE STUDY

The general aim of this study was to investigate the role of complement (C) activation and regulation in chronic mucosal infections.

The specific goals were to analyze:

I C activation and the expression of C regulators in chronically infected mucosa *in vivo* (in adult periodontitis and *H. pylori*-gastritis),

II whether gram-negative bacteria causing chronic infections and persisting for long periods on mucosa can acquire the host cell C inhibitor CD59 onto their surfaces and thereby prevent MAC attack and,

III how a viral (HSV-1) infection of neuronal and epithelial cells affects their susceptibility to C attack *in vitro*.

7 MATERIALS AND METHODS

7.1 MATERIALS

7.1.1 Patients

7.1.1.1 Adult periodontitis (AdP, I)

The patients who participated in study I received surgical treatment at the Department of Periodontology or Oral Surgery at the Institute of Dentistry, University of Helsinki. Two additional AdP samples were obtained at autopsies at the Department of Pathology, University of Helsinki, 1-2 days *post mortem*. The study was approved by the ethical committee of the Institutes of Dentistry, University of Helsinki, and the subjects were enrolled into the study and treated in compliance with the Helsinki Agreement as revised in 1983.

Samples of healthy gingiva (controls) were obtained from 11 individuals undergoing operative extractions of clinically noninflamed, partially erupted third molars. The controls had no radiographic evidence for loss of tooth attachment and the maximum pocket depth was 3.5 mm. Samples of diseased gingiva (AdP) were collected from 18 patients with moderate to severe generalized adult periodontitis as judged by clinical measurements of pocket depths, loss of attachment, radiographic bone loss, suppuration and gingival bleeding on probing. AdP patients chosen had radiographic evidence for bone loss of 20 - 50% on many teeth and a mean loss of attachment ranging from 4 to 6 mm. Samples were obtained from premolar-molar regions during flap surgery of the initial periodontal therapy. All sample sites were greater than 6 mm in probing depth and bled on probing. Results on the clinical examination and status of patients are described in article I.

7.1.1.2 *H. pylori*-gastritis (III)

Gastric biopsies were obtained from a total of 60 (30 men, 30 women) patients undergoing routine diagnostic gastroscopic examination at the Helsinki University Central Hospital in 1998 and 2000. Upper gastrointestinal endoscopies were performed with Olympus GIFQ-100 video endoscopes. Three biopsy specimens were obtained; two from the corpus and one from the antrum. Standard histology was performed from antrum and corpus biopsies at our University Central Hospital Laboratory Diagnostics Unit (Department of Pathology). *H. pylori* infection was determined by standard histology and by staining the corpus samples with a mAb against *H. pylori*. The examination of the histological specimens is described in article III.

Ten patients were found to have *H. pylori* infection (5 men and 5 women; age range 26-86 years, mean 61.8 years) and six histologically *H. pylori* negative patients were chosen as controls (1 man and 5 women; age range 21-71 years, mean 59.4 years). The subjects were enrolled into the study and treated in compliance with the Helsinki Agreement as revised in 1983. The sampling from the patients was approved by the Ethical Committee of the Department of Surgery at the Helsinki University Central Hospital.

7.1.1.3 Tissue preparation

The biopsy samples for indirect immunofluorescence were cleaned of macroscopic blood by rinsing with 0.9% NaCl and taken to the laboratory on an ice bed. Samples were snap frozen using liquid nitrogen within 30 minutes after biopsy and stored at -70°C during the sample collection. For the immunohistochemical analyses the samples were sectioned in a cryostat (4 µm) and stored at –20°C until used.

7.1.2 Bacteria

For the experiments using *E. coli* (II) strains EH237 (LPS chemotype Re)[167], EH234 (LPS chemotype Ra)[168] and an encapsulated strain IH3080 (O18:K1:H8)[169] were used (by the courtesy of Dr. M. Vaara at our department). The luciferase gene expression vector pCSS962 (lucGR)[170,171] and a helper plasmid pGB3[172] were kindly provided and cloned into the *E. coli* strains EH234 and JM103 (LPS chemotype Ra) by Dr. M. Karp at the University of Turku, Finland. Bacteria were grown at 37°C on Luria broth. Following overnight growth, the bacteria were washed three times (1750*g*, 8 min), resuspended into buffer and concentrated to approximately 2 x 10^{10} bacteria/ml for binding analyses.

For the *H. pylori* experiments (III) strain NCTC 11637 ($CagA^+$) and two fresh clinical isolates 9:0 ($CagA^+$) and 67:20 ($CagA^-$, by the courtesy of Professor L. Engstrand, Karolinska Institute, Stockholm, Sweden) were cultured on Brucella agar plates (BBL, Cockeysville, MD) supplemented with whole horse blood (7%). The plates were incubated for 48 hours at 37°C in a microaerophilic atmosphere generated by Anaerocult C (Merck, Darmstadt, Germany). The bacteria were collected, washed three times (1750*g*, 8 min), resuspended into buffer and adjusted to yield an OD value of 1.0 at 650 nm. Finally, the bacteria were concentrated to approximately 2 x 10^{10} bacteria/ml.

7.1.3 Eukaryotic cell culture

The Paju tumor cell line[173] was established from a pleural metastasis of a neural-crest-derived tumor (kindly provided by Prof. L. C. Andersson at the Department of Pathology at our Institute). The Paju cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin G (10 U/ml), streptomycin sulfate (50 mg/ml)

and 1 mmol/L glutamine. Human embryonal skin (HES) fibroblasts were prepared using conventional methods from an embryonal skin specimen (initiated by Prof. A. Vaheri at the Department of Virology at our Institute). For subculturing, the cells were detached by treatment with Versene/EDTA (Life Technologies Inc., Grand Island, NY). For the experiments the cells were cultured in 24-well plates on microscope glass cover slips for 48 hours.

7.1.4 HSV-1 infection

The HES and Paju cells were infected with *Herpes simplex* -virus type 1 (15 pfu/cell). After incubating for 1 hour the cells were washed twice with PBS and once with the RPMI 1640 medium to remove the extracellular virus. To neutralize the viruses 0, 1, 2, 3, 5, 7 or 12 hours after infecting the cells they were treated (10 min at -20°C) with acetone. Finally, the cells were washed three times with phosphate buffered saline (PBS; pH 7.4) and stored at 4°C until immunostained.

7.1.5 Buffers

Veronal buffered saline (VBS; 3.2 mM diethyl barbituric acid, 1.8 mM diethyl barbituric acid sodium salt, 0.15 M NaCl, pH 7.3), VBS containing $MgCl_2$ (0.5 mM) and $CaCl_2$ (0.15 mM) (VBS^{++}) or phosphate buffered saline (PBS; pH 7.4) were used as buffers. In some instances, 0.05% BSA or 0.05% Tween 20 (T) was added to VBS (VBS-BSA), VBS^{++} (VBS^{++}-BSA) or PBS (PBS-T) to reduce nonspecific reactions. In the whole cell ELISA, 0.5% BSA in VBS was used as a blocking reagent. For the CD59 binding assay (articles II, III) stock solutions of $CaCl_2$ and $MgCl_2$ were prepared into distilled H_2O. The cations were diluted into VBS to obtain eight final concentrations ranging between 0-30 mM.

7.1.6 Reagents and sera

The antibodies and the sera were used as described in detail in the individual articles. A summary is presented in tables 3-6.

Table 3. Antibodies against complement components

Ab	Type	Source	Method*	Used in
C1q	pAb, rabbit	Dakopatts, Glostrup, Denmark	IF	IV
C3c	pAb, rabbit	Dakopatts, Glostrup, Denmark	IF	III, IV
C3d	pAb, rabbit	Behringwerke AG, Marburg, Germany	IF	I
C4c	pAb, rabbit	Dakopatts, Glostrup, Denmark	IF	IV
C9	pAb, goat	Quidel, San Diego, CA	IF	I
C5b-9	mAb IgG2a, mouse	Quidel, San Diego, CA	IF, WC-ELISA	II, III, IV

Table 4. Antibodies against complement regulators

Ab	Name	Type	Source	Method*	Used in
DAF (CD55)	BRIC213	mAb IgG2b, mouse	Int. Blood Group Ref. Lab., Elstree, UK	IF	III, IV
MCP (CD46)	GB24	mAb IgG1, mouse	Dr. K. Liszewski and Prof. J. P. Atkinson, Washington Univ. School of Med., St. Louis, MO, USA	IF	IV
CD59	BRIC229	mAb IgG2b, mouse	Int. Blood Group Ref. Lab., Elstree, UK	IF, FACS, WC-ELISA	I, II, III, IV
	YTH53.1	biotinylated mAb IgG2b, rat	Prof. H. Waldmann, Sir William Dunn School of Pathology, Oxford, England	Double IF	III
Vitronectin		pAb, rabbit	Behringwerke AG, Marburg, Germany	IF	I

***** IF, immunofluorescence microscopy; FACS, Flow cytometric cell sorting; WC-ELISA, whole cell enzyme-linked immunosorbent assay

Table 5. Antibodies against microbes and control antibodies

Ab	Type	Source	Method*	Used in
H. pylori	mAb IgG2a, mouse	Orion Diagnostica, Espoo, Finland	IF, WC-ELISA	III, IV
HSV-1	mAb IgG, mouse	Biodesign, ME, USA	IF	IV
rat podocalyxin	mAb, mouse	Dr. A. Miettinen at our department	IF	I
anti-idiotype AF1	mAb IgG, mouse	Dr. M. Kaartinen at our department	IF	II
anti-idiotype AF-16.1	mAb IgG1, mouse	Dr. M. Kaartinen at our department	IF	II, III
anti-idiotype 1.5.2	mAb IgG2b, mouse	Dr. M. Kaartinen at our department	IF	III

Table 6. Secondary antibodies

Ab	Source	Method*	Used in
FITC anti-mouse IgG	Dakopatts, Glostrup, Denmark	IF	I
	Jackson ImmunoResearch Laboratories, West Grove, PA	IF	II, III
	Alexa Molecular Probes, Eugene, OR, USA	IF	IV
FITC anti-rabbit IgG	Dakopatts, Glostrup, Denmark	IF	I
	Jackson ImmunoResearch Laboratories, West Grove, PA	IF	III
	Alexa Molecular Probes, Eugene, OR, USA	IF	IV
FITC anti-goat IgG	Dakopatts, Glostrup, Denmark	IF	I
Rhodamine neutravidin	Alexa Molecular Probes, Eugene, OR, USA	Double IF	III
POX anti-mouse IgG	Jackson ImmunoResearch Laboratories, West Grove, PA	WC-ELISA	II, III

***** IF, Immunofluorescence microscopy; WC-ELISA, whole cell enzyme-linked immunosorbent assay

Normal human serum (NHS) samples were obtained from healthy laboratory personnel and kept frozen in aliquots at –70°C. In study IV sera obtained from laboratory personnel with IgG-class antibodies against HSV-1 were used as immune sera (NHSimm). Sera from personnel without history of HSV infections and no antibodies against HSV-1 or HSV-2 of IgG- or IgM-class were used as nonimmune sera (NHSni). The HSV-1 antibodies were detected with a standard ELISA method at the Helsinki University Central Hospital Laboratory Diagnostics (Helsinki, Finland). Sera were heat-inactivated (NHShi) by treatment at 56°C for 30 min. The sera were kept frozen in aliquots at –70°C.

7.1.7 Isolation of CD59

A glycolipid-tailed membrane form of CD59 (mCD59) was purified from human erythrocytes and a soluble form (sCD59) from human urine. YTH53.1-Sepharose 4B affinity chromatography was used as described earlier[174]. The used rat mAb YTH53.1 (IgG2b) against CD59 was obtained from Prof. H. Waldman, Dept. of Pathology, University of Oxford, UK. Bound proteins were eluted with 100 mM glycine buffer, pH 2.5, into tubes containing 50 µl of 1M Tris buffer, pH 9.0, pooled and dialyzed against VBS. During purification mCD59 contained 0.1% NP40 detergent. The purity of the CD59 preparations was confirmed by SDS-PAGE and was found to be over 80%. The protein concentrations were analyzed by the BCA Protein Assay kit (Pierce Chemical Co., Rockford, IL, USA).

Both mCD59 and sCD59 were radiolabeled with Na[^{125}I] using the Iodogen method (Pierce Chemical Co., Rockford, IL, USA). The ^{125}I-mCD59 (initial activity: $2x10^7$ cpm/µg) stock preparation contained 0.02% NP-40 detergent. ^{125}I-sCD59 (10^7 cpm/µg) did not contain detergent but for comparative binding assays it was reconstituted with NP40 at concentrations equivalent to those used with ^{125}I -mCD59.

7.2.1 METHODS

7.2.1 Indirect immunofluorescence microscopy (IF)

Frozen sections or bacteria air-dried on microscope slides were fixed in cold acetone (-20°C) for 10 minutes and prewashed with PBS-T or PBS-BSA. The sections were incubated for 30 minutes at +22°C in a humid chamber with the different primary antibodies (Tables 3-5) at concentrations described in individual articles. Control incubations were performed either by replacing the primary antibody with buffer or with an irrelevant isotype-matching antibody at an equivalent concentration (Table 5). After rinsing three times with buffer the sections were incubated for 30 minutes at +22°C with fluorescein isothiocyanate (FITC)-conjugated antibodies against mouse, rabbit or goat immunoglobulins, respectively, or with the rhodamine neutravidin conjugate for biotin detection (Table 6). After rinsing again three times the samples were mounted in Mowiol (articles I-III) or in the Immu-Mount™ mounting medium (Shandon Inc., Pittsburgh, PA, USA), (article IV).

The samples for immunofluorescence-microscopy were examined using a Zeiss (article I) or an Olympus BX50 (articles II-III) standard microscope with a filter specific for FITC-fluorescence. Sections were photographed with a Nikon automatic camera system using Kodak Tri-X 400 film. In study IV a Hamamatsu $ORCA_{IIIm}$ digital color camera (Hamamatsu Photonics, Hamamatsu City, Japan) together with the Openlab 2.2.5 imaging application (Improvision, Coventry, UK) was used for photography.

7.1.8 CD59 binding tests

Three different strains of *E. coli* (I) and three different strains of *H. pylori* (III) were incubated with four different concentrations of ^{125}I-mCD59 and ^{125}I-sCD59 (0.5 ng-0.5 µg/10^9 bacteria in a final volume of 100 µl of VBS-BSA) at 37°C for 30 min with gentle shaking. To study the effect of divalent cations on the CD59 binding, the bacteria were incubated with ^{125}I-mCD59 or ^{125}I-sCD59 (0.4 µg/10^9 bacteria in a final volume of 100 µl) in the presence of eight different concentrations (final concentrations 0-30 mM) of Ca^{++} or Mg^{++}. After washes, the cell-bound and free ^{125}I-CD59 were separated by centrifuging (5000*g*, 1 min) the mixtures through 20% sucrose (250 µl) in narrow (0.4 ml) test tubes. The bottom parts of the tubes containing the cells were cut out, and radioactivities in both pellets and supernatants were counted. To control for possible cation-induced precipitation of CD59 the binding experiments were repeated in the absence of bacteria.

7.1.9 Flow cytometry

For FACS analysis (II) the *E. coli*-bacteria were resuspended to approximately 10^9 bacteria/ml. mCD59 was allowed to bind to the EH237 bacteria as described above. After washing three times with VBS-BSA, the bacteria were incubated for 30 min at 37°C with the BRIC229 Ab (3.3 µg/ml) against CD59. After washing, the secondary FITC-conjugated Ab was added and the bacteria were incubated at 37°C for 30 min, washed three times and examined immediately by flow cytometry. Control stainings were performed by omitting CD59 or the primary Ab. All samples were examined by a Becton Dickinson FACScan 440 (San Jose, CA, USA) flow cytometer with an argon laser tuned to 488 nm at a power output of 15 mW. The data were analyzed with the Lysys II software supplied by Becton Dickinson.

7.1.10 Bactericidal assay

The serum sensitivity of the *E. coli* and *H. pylori* -bacteria was examined with the bactericidal assay. The bacteria were incubated with or without mCD59 (0.01 µg/2x10^5 bacteria in 100 µl of VBS for 30 min at 37°C) as described above. After three washes with VBS^{++}-BSA the *E. coli* were incubated with 0, 1.7, 5 or 17% of NHS or NHShi in a final volume of 500 µl. After washing, serial 10-fold dilutions of bacterial suspensions were made into saline and 900 µl of each dilution of was plated on Luria agar plates. The washed *H. pylori* were incubated with 0, 1.7, 3.3 or 5.5% of NHS or NHShi and the 10-fold dilutions were plated on Brucella-agar plates. After 15-hour incubations at 37° C the colony forming units (CFUs) were counted. The survival of bacteria in NHS was calculated relative to that in NHShi.

7.1.11 Bioluminescence assay for bacteriolysis

The serum sensitivity of the EH234 and JM103 strains of *E. coli* was also studied using a bioluminescence method. A structural gene for luciferase from the Jamaican click beetle *Pyrophorus plagiophthalamus* was cloned and expressed in the two strains of *E. coli*. Incubations with mCD59 (0.4 µg/10^9 bacteria in 1 ml) and NHS were performed as described above. In controls, mCD59 was omitted or NHShi was used. The JM103 strain and the bioluminescence method used have been described earlier[175,176]. Briefly, live bacteria expressing the luciferase enzyme illuminate in the presence of the luciferine substrate. Bacterial death leads to the loss of enzyme production and activity resulting in a decrease in illumination. The substrate, 0.25 mM D-luciferine in sodium citrate, pH 5.0, was added 90 minutes before measuring the luminescence. Luminoskan EL1 luminometer and Biolise software (Labsystems, Helsinki, Finland) were used for data collection and analysis.

7.1.12 Whole cell ELISA

A modification of a whole cell ELISA method for C3 deposition onto mycobacteria[177] was used for analysis of CD59 binding and C5b-9 complex formation on *E. coli* and *H. pylori*. EH237 and IH3080 strains of *E. coli* and 11637, 9:0 and 67:20 strains of *H. pylori* were incubated with or without mCD59 (2 µg/10^9 bacteria in a final volume of 2 ml of VBS^{++}) in the presence of 2.5 mM Ca^{++}. The bacteria were washed with VBS^{++}-BSA and divided into aliquots. *E. coli* were incubated with 0, 1.7, 5 and 17% and *H. pylori* with 0, 1.7, 5 and 17% of NHS or NHSi in a final volume of 500 µl. The bacteria were then washed again and resuspended to 10^9 bacteria/ml. 50 µl aliquots were dispensed into the wells of microtiter plates and allowed to dry overnight at 37°C. Each well was washed three times with PBS-T, incubated with 75 µl of 0.5% BSA in PBS for 1 h at 37°C to block nonspecific protein binding sites and washed again. Six replicate wells for each combination of experimental conditions (EH237 or IH3080 of *E. coli* with or without mCD59, four concentrations of NHS or NHShi and 11637, 9:0 and 67:20 of *H. pylori* with or without mCD59, four concentrations of NHS or NHShi) were incubated for 1 h at 37°C with or without 50 µl of the primary antibody against CD59 or the C5b-9 neoepitope or *H. pylori* in article III. The wells were washed and the secondary peroxidase-conjugated Ab was added. The plates were incubated for 1 h at 37°C and washed thoroughly with PBS-T. Phenylenediamine dihydrochloride substrate in ureoperoxidase (Dakopatts, Glostrup, Denmark) was added and the reaction was stopped after 5 min with 50 µl of 20% sulfuric acid. The absorbances were read using a 492-nm filter on an ELISA reader (Labsystems Multiskan MCC/340, Helsinki, Finland). Absorbances in wells with NHShi-incubated bacteria were subtracted as background in the C5b-9-ELISA and absorbances in wells with CD59-uncoated bacteria were subtracted as background in the CD59-ELISA. In study III the ODs were

calibrated with the amount of *H. pylori* in each row (OD of *H. pylori* wells). The means and standard deviations of the absorbances for the replicate wells of each type were calculated.

7.1.13 SDS-PAGE

The purity of the purified CD59 was examined by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). It was performed with a 12% mini-gel (BioRad Laboratories, Richmond, CA, USA) under reducing conditions. The reducing conditions were achieved by using 5% β-mercaptoethanol. The gels were stained with Coomassie brilliant blue which showed bands of 18 to 21 kDa for CD59 under reducing conditions.

7.1.14 Statistical methods

The significance of the decrease in C5b-9 formation by binding of CD59 was examined by the two-tailed Student's t-test (II, III). The correlation between bound CD59 and C5b-9 neoepitope expression was analyzed by linear regression (II, III).

8 RESULTS AND DISCUSSION

8.1 ADULT PERIODONTITIS (AdP)

8.1.1 Complement activation in AdP

The C3 (C3d) and C9 components of complement were observed to be deposited on the basement membranes (BM) of both control and AdP gingival epithelium. The connective tissue of the control gingiva (n=11) was mainly negative for C3 and C9 whereas evidence for distinct C activation could be detected in the connective tissue of the AdP gingiva (n=18). Coarse granular deposits of C3 were detected below the BM in two thirds of the AdP cases and a similar or more diffuse staining for C9 was seen in one third of the AdP cases. The presence of C deposits in the connective tissue indicates that, in addition to the previously described fluid phase cleavage of C components in AdP[73], C activation occurs also in the subepithelial regions.

In addition to the subepithelial deposits of C components in AdP patients linear deposits of both C3d and C9 were observed along the BM of both pocket and oral epithelium in healthy and diseased gingiva. This finding is analogous to the staining of C components in other human tissues e.g. in the skin[178] and in the kidney glomerulus[179]. The C system, especially the alternative pathway, readily recognizes and attacks foreign material entering human tissues. To accomplish this the C system must be active at a low level all the time. BMs are thin extracellular sheets that, in addition to giving mechanical support for the overlying tissues, act as semipermeable barriers. In gingiva BMs constantly filter plasma to generate the gingival crevicular fluid (GCF). Thereby the BMs also become exposed to C components. The presence of C3d on the BMs of kidney glomeruli[179], and in gingiva as observed here, could thus

be interpreted to be the result of a normal C turnover process. Complement deposits in the connective tissue beneath the BM, however, probably reflect disease associated C activation. This could be a result of insufficient C regulating activity in this region.

8.1.2 Complement regulation in AdP

In gingival tissues of AdP patients the C components C3d and C9 seemed to be present at the same sites as vitronectin (VN), a plasma inhibitor of the terminal C cascade. The presence of VN in these deposits is in accordance with previous findings that vitronectin colocalizes with components of MAC in glomerulonephritis[21] and in myocardial infarction [180,57]. C activation products deposited on the BM together with vitronectin. The inefficiency of vitronectin to prevent the deposition of MAC has been considered to be due to its other functions, especially in the clearance of cell debris after tissue injury. Vitronectin could thus participate in the clearance of nonviable cells from tissues by macrophages. The demonstration of the $\alpha_v\beta_3$-integrin receptor for vitronectin on osteoclasts[181] suggests that vitronectin could also be involved in bone remodeling through the activation of osteoclasts after binding of vitronectin-MAC complexes to osteoclast vitronectin receptors.

The MAC inhibitor CD59 was found to be strongly expressed in the epithelium of healthy human gingiva and present in the AdP gingiva. No C components were seen in areas of CD59 expression. This indicates that the gingival epithelium is well protected against MAC-mediated cell damage. The expression of CD59 in AdP was different from that in the healthy gingiva. The staining of the epithelium, especially of the pocket epithelium, for CD59 was weaker in AdP and often granular. The granular expression of CD59 in the AdP epithelium could be due to a detachment or shedding of the phospholipid-tailed form of CD59 from cell membranes. Somewhat similar observations have been made e.g. in previous studies of myocardial infarction[182]. Protectin was strongly expressed by

endothelial cells in both control and AdP gingival blood vessels. In a previous study[183] the expression of CD59 in cultured endothelial cells was shown to be up-regulated up to threefold by proinflammatory activators of cellular second messenger stimuli. In AdP a possible increase in the vascular expression of CD59 may not have become visualized because of an already strong expression in healthy endothelial cells.

The active form of CD59 has a glycophospholipid-tail (GPI) but the phospholipid part is lost from the soluble form of CD59[35, 41, 184]. The GPI-anchor may not only provide increased lateral mobility for membrane proteins[184] but it may also allow the proteins to become released from cell membranes. The granular expression pattern of CD59 suggests that an inflammatory reaction and tissue damage may induce instability of GPI-anchored molecules on the cell membranes. The decrease in the expression of CD59 in AdP epithelium may also result from an inability of the epithelial cells to upregulate CD59 expression or to maintain the synthesis of GPI-anchored proteins during an enhanced turnover. The GPI-anchored proteins released into the fluid phase may become incorporated in a functionally active form into new cell membranes. Shedding could be a practical and economical way of mobilizing useful proteins from nonviable tissues for later use elsewhere.

The results of study I suggest that if periodontal inflammation reaches connective tissue near the alveolar bone it is not protected against MAC attack. The regulation of MAC formation and prevention of tissue damage takes place in the epithelium of the periodontium and in the endothelium of the blood vessels of the underlying connective tissue. The epithelium is not as strongly affected as the underlying connective tissue and the progression of the disease apparently takes place in the deeper, more vulnerable, parts of the gingiva.

8.2 BINDING OF CD59 TO BACTERIA *IN VITRO*

8.2.1 Binding of CD59 to *E. coli*

The fact that CD59 can be released from cell surfaces suggested that the active form of CD59 could incorporate into outer cell membrane of a microbe and increase its resistance to MAC-mediated lysis. The ability of CD59 to bind to the surface of gram-negative bacteria was examined directly using ^{125}I-labeled CD59 in a binding assay. In the absence of added divalent cations, phospholipid-tailed CD59 bound to the non-encapsulated *E. coli* strain EH237 (Re chemotype) but not to the non-encapsulated strain EH234 (Ra chemotype) or to the encapsulated strain IH3080 (II). Protectin binding was dependent on the glycolipid moiety since no binding of soluble CD59 (sCD59) to any of the *E. coli* strains was observed. The addition of increasing concentrations of Ca^{++}, but not of Mg^{++}, resulted in a significant increase in the binding of phospholipid-tailed CD59 to both non-encapsulated strains (EH234 and EH237). A detectable increase in binding occurred at 0.5 mM Ca^{++} and at a near physiological concentration of Ca^{++} (2.5 mM) an average of 50% of the lipid-tailed CD59 bound to the non-encapsulated strains. Ca^{++} did not induce binding of mCD59 to the encapsulated strain IH3080. Indirect immunofluorescence microscopy showed that bacteria incubated with mCD59 in the presence of Ca^{++} stained positive for CD59 whereas in the absence of Ca^{++} the staining was clearly less intense. Flow cytometric analysis also showed that mCD59 bound to the bacteria.

Results of study II showed that outer cell membranes of *E. coli* can act as acceptors for GPI-anchored proteins. mCD59 bound to non-encapsulated *E. coli* in the presence of Ca^{++}-ions and protected the bacteria against C lysis. The persistence of bound CD59 on the bacteria and the inability of the soluble CD59 to bind to the bacteria, it is likely that the association was due to a hydrophobic interaction between the glycolipid-tail and the lipid A-rich bacterial

outer cell membrane. Since human cell membranes can analogously bind only phospholipid-tailed CD59, it is probable that the phospholipid moiety becomes incorporated into the bacterial outer membrane in a manner similar to eukaryotic cell membranes. The amount of CD59 offered to *E. coli* was estimated to result in a density similar to that on cultured human endothelial cells[183,185]. Increasing the concentration of Ca^{++} increased the density of mCD59 in the bacterial outer membrane. A subsequent decrease in the proportion of lipid A on the membrane is, however, likely to cause instability of the membrane.

A capsule prevented the binding of GPI-anchored CD59 to the bacteria. It forms a steric barrier on the surface of the bacterium and the probability of a lipid-rich micelle to pass the barrier and enter the outer membrane is low. A long oligosaccharide side-chain of LPS also seemed to hinder the binding of CD59 to the bacteria in the absence of exogenously added Ca^{++}-ions, suggesting partial steric hindrance by the long oligosaccharide. This also indicates that the bacterial outer membrane rather than the oligosaccharide side-chain is the principal mCD59 binding structure. The role of CD59 as a receptor for the bacteria examined was ruled out by the fact that no binding of the soluble form of CD59 was observed.

8.2.2 Binding of CD59 to *H. pylori*

Since study II indicated that *Escherichia coli* was able to bind mCD59 it was next (III) considered that this could be a more common property of gram-negative bacteria. Indirect immunofluorescence staining analysis of biopsy samples from patients with *H. pylori* gastritis showed that the *H. pylori* seen in the gastric pits often stained positive for CD59. Since this suggested acquisition of CD59 by *H. pylori in vivo* it was tested whether CD59 bound to the surface of *H. pylori in vitro*. This was first examined using ^{125}I-labeled CD59 in a direct binding assay. On average, *H. pylori* strain 11637 bound 12-

18 % of the phospholipid-tailed CD59 offered. The CagA positive clinical isolate 9:0 bound 16-17% and the CagA negative isolate 67:20 bound 10-12% of the mCD59 offered. The amount of mCD59 bound to the 67:20 isolate was significantly smaller ($p < 0.005$) compared to the two CagA positive strains. The difference between the two CagA positive strains was not significant. As with *E. coli* CD59 binding was dependent on the glycolipid-moiety since no binding of soluble urinary CD59 (sCD59) to *H. pylori* was observed. In contrast with *E. coli*, CD59 binding to *H. pylori* was only slightly enhanced by the divalent cation Ca^{++}. Mg^{++} had no effect on the binding.

The *H. pylori* strains studied bound significantly less mCD59 than *Escherichia coli* under similar conditions. This could be explained by differences in the structures of the outer cell membranes and in the outer surface area per bacterium. The 10-18% binding still results in approximately 40,000 - 75,000 molecules per bacterium. Unlike with *E. coli*, the binding was only weakly enhanced by Ca^{++} which also may be due to a different organization of LPS and other outer membrane structures. As with *E. coli,* the binding of CD59 to *H. pylori* was dependent on the phospholipid-tail since no binding of the soluble urinary form of CD59 was observed.

8.3 INHIBITION OF C BY BINDING OF mCD59 TO *E. COLI* AND *H. PYLORI*

The ability of bacteria-bound mCD59 to prevent C-mediated bacteriolysis was studied by counting viable bacteria after exposure to increasing concentrations of NHS. The binding of mCD59 increased the resistance of both *E. coli* strains (II) and all three *H. pylori* strains (III) against active human serum. 50% of the *E. coli* EH237 with bound mCD59 survived an exposure to 1.0% NHS compared with only 12% survival of the native bacteria. The *E. coli* with bound mCD59 needed at least three times higher serum concentrations than native bacteria for 50% killing. The increase in C resistance of *H. pylori* was statistically significant for the CagA positive strains

11637 and 9:0 when serum concentrations up to 1% ($p < 0.01$) or 3% ($p<0.05$) were used, respectively. The difference was not significant for the CagA negative 67:20 isolate.

The ability of bacteria-bound mCD59 to prevent C-mediated bacteriolysis was also studied by measuring changes in the luminescence of luciferase-transfected bacteria (II). In the bioluminescence assay more than 90% of the *E. coli* EH234 with bound mCD59 survived the exposure to 1.0% NHS compared with 50% survival of the native bacteria. The bacteria with bound mCD59 needed at least twice the amount of serum as native bacteria for 50% lysis.

The ability of membrane-bound mCD59 to prevent deposition of the C membrane attack complex was studied with a whole cell ELISA. After treatment with serum at concentrations up to 3%, the mCD59-coated *E. coli* expressed significantly less ($p<0.05$) C5b-9 neoepitope than the uncoated bacteria. C5b-9 deposition correlated negatively with the amount of mCD59 binding to the EH237 strain ($r=-0.973$, $p<0.05$). The mCD59-coated CagA positive *H. pylori* expressed significantly less ($p < 0.05$) C5b-9 neoepitope than the uncoated bacteria when 5% serum was used. The bacteria-bound mCD59 did not have a similar effect on the CagA negative strain 67:20. C5b-9 deposition correlated negatively with the amount of mCD59 bound to the *H. pylori*. The correlation was statistically significant ($p < 0.01$) only for the CagA positive strains 11637 and 9:0.

Bacteria-bound mCD59 was found to protect the bacteria against complement-mediated lysis *in vitro*. In accordance with inhibiting bacteriolysis, the bacteria-bound CD59 inhibited the expression of the C5b-9 neoepitope on the bacteria. This further suggests that CD59 inhibited the MAC similarly as on eukaryotic cells. A difference was seen between the CagA positive and negative *H. pylori* strains. The CagA negative strain bound less CD59 and appeared to benefit less from CD59 binding to it. This finding could be related to the

observations that CagA positive strains of *H. pylori* are often found deep in the gastric pits and in closer contact with the gastric mucosa whereas CagA negative strains colonize more superficial parts[186].

8.4 COMPLEMENT ACTIVATION AND REGULATION IN *H. PYLORI* GASTRITIS

8.4.1 Activation of C in *H. pylori* gastritis

Activation products of complement C3 were observed to be deposited on the surface and below the gastric epithelia of both *H. pylori*-infected and noninfected patients (III). The staining was often granular and more intense in the *H. pylori*-infected mucosa than in the controls. Positive staining for C3 could also be seen around connective tissue blood vessels. The *H. pylori* bacteria seen in the superficial mucus were often opsonized with C3 and coccoid in shape. The mucus of the gastric pits and the bacteria there were often negative for C3.

In general, the staining for the C5b-9 neoepitope was positive in the same areas as the staining for C3. C5b-9 was observed to be deposited around blood vessels and below the epithelia of both *H. pylori*-infected and noninfected gastric mucosa. In the *H. pylori*-infected mucosa the staining for C5b-9 was often granular and more intense than in the controls. The *H. pylori* seen in the superficial mucus were often positive for C5b-9 and coccoid in shape. The mucus of the gastric pits and the *H. pylori* there were in most cases negative for C5b-9.

Evidence for local mucosal C activation was seen in patients with *H. pylori* gastritis but also to some extent in patients without *H. pylori* infection. The presence of C activation products could thus in part be due to a normal C turnover process. It is also possible that other activators of C are present in the gastric mucosa.

8.4.2 Regulation of C in *H. pylori* gastritis

The glycolipid-tailed C inhibitors DAF and CD59 were found to be expressed on the epithelial and endothelial cell membranes of the gastric mucosa (III). In general, the staining for CD59 was more intense than for DAF and the vascular endothelia stained most prominently. The staining of the epithelial cells for CD59 was often apically granular or less intense in the infected mucosa, especially in the gastric pits. The staining for DAF, however, was intensified in infected mucosa. The gastric mucus of the patients, but not of the controls, was in most cases positive for CD59 and DAF suggesting shedding or release of the proteins.

The granular staining pattern and shedding of CD59 to the mucus was found to be typical for *H. pylori* infection. *H. pylori* produces a phospholipase PldA[187], which could be responsible for sensitizing local host tissue to complement activation during *H. pylori* infection. On the other hand, the endogenous phospholipase A_2 from neutrophils may induce mucosal tissue to lose its phospholipid-anchored regulators or the ability to bind factor H. Unlike phopholipases C and D, phospholipase A_2 and the *H. pylori* phospholipase PldA may cause a more general perturbation of the cell membrane and release of host cell GPI-anchored proteins together with their anchor phospholipids[188]. This could make the mucosa susceptible to complement-mediated damage. It is likely that the closer a bacterium is to the host tissue the more instability it causes and the more it is exposed to the C system. Shedding of CD59 together with its GPI-lipid-moiety may allow its incorporation into neighbouring cells, including bacteria.

The *H. pylori* seen in the gastric pits stained often positive for CD59 whereas bacteria outside the pits were negative. In areas where the *H. pylori* bacteria stained positive for CD59 they stained negative for C5b-9. This suggests that the transferred CD59 had been functional. No positive staining of *H. pylori* for DAF could be detected although

the staining for DAF on the mucosal cells and mucus was intensified in the infected mucosa. The simultaneous incorporation of the two GPI-anchored C regulators, CD59 and DAF could result in a further increase in the C resistance of the bacteria.

8.5 COMPLEMENT ACTIVATION AND REGULATION BY HSV-1-INFECTED EPITHELIAL AND NEURONAL CELLS

To analyze the effects of a viral infection on complement regulator expression human epithelial HES and neuronal Paju cells were infected with the Herpes simplex virus type 1 (HSV-1) (IV). When the noninfected cells were exposed to C in fresh human serum the cells did not show any specific, positive staining for the C components studied on either cell type. After infecting HES cells with HSV-1 they soon began to activate C. Exposure to non-immune serum resulted in weak, granular C3, C4 and C5b-9 deposits three to five hours after infecting the cells and C1q deposition could be detected at the 12-hour time point. When the infected HES cells were exposed to immune serum, C1q, C3, C4 and C5b-9 deposits were first detected at the 1 and 2 -hour time points. By 5 hours nearly half of the HES cells had deposited C components. Twelve hours after infecting the cells nearly all of them stained positively for early C components and three fourths of cells were positive for C5b-9. Initially, the positive staining of individual HES cells for C components was seen mainly on the contracted cells and cytoplasm adjacent to the nucleus but by 12 hours all cells stained positively throughout. In addition, the exposure to immune serum resulted in a spindle-shaped morphology and a further reduction in cell size.

When the HSV-1-infected Paju cells were exposed to nonimmune serum, weak, granular C3 and C4 deposits could be detected three to five hours after infecting the cells but no C1q or C5b-9 deposits were seen at any time point. This suggested weak activation of the AP. When the Paju cells were treated with immune serum weak C component deposition was detectable within one hour after

infecting the cells. By two hours one tenth of the cells had bound C1q and C4 and one fourth of the cells were positive for C3. Less than one tenth of the Paju cells were positive for C5b-9 at the same time point. The staining intensity and the percentage of Paju cells positive for C1q, C3, C4 and C5b-9 decreased by three hours. By 12 hours one third of the Paju cells stained positive for C1q and C4, nearly half for C3 and only one tenth for C5b-9.

Patients with recurrent HSV-1 infections normally develop antibodies against HSV-antigens capable of activating the classical pathway. In the fourth study HSV-1 infection rapidly resulted in HSV protein expression on the surfaces of both HES and Paju cells. In contrast to HES cells, the expression of viral antigens on Paju cells started to decrease after 3 hours. At the 12-hour time point all HES cells, but only 40 % of the Paju cells, expressed viral antigens. This suggested that the infection became latent in the neuronal cells. Despite their C-activating potential only 10 % of Paju cells deposited terminal C components from immune serum during the 12-hour follow-up. This indicated that C activation was relatively well controlled on this cell type.

Intact HES cells stained weakly positive for MCP (CD46). The staining was evenly distributed over the epithelial cells. The expression was down-regulated three hours after infecting the cells. Intact Paju cells did not express MCP but HSV-1 infection induced expression which lasted for approximately 3 hours. The glycolipid-tailed regulator DAF (CD55) was expressed on intact HES and Paju cells. The staining for DAF was markedly intensified during the first 3 to 5 hours of the HSV-1 infection. Similar increase in intensity was observed in the inflamed epithelia of the periodontium (I) and gastric mucosa (III). DAF staining was located to cell membranes and the contracted HES cells, and the shrunk extensions of Paju cells stained strongly positive. During the next few hours the staining intensity for DAF decreased in both cell types and was totally lost in HES cells by 12 hours. In contrast, the Paju cells remained positive for DAF at all the time points studied.

CD59 was strongly expressed on intact HES and Paju cells and the staining was evenly distributed on the cell membranes. No change in the overall staining intensity for CD59 was detected during the first 5 hours of infection in either cell type. The contracted extensions of the Paju cells stained strongly positive for CD59. Seven hours after infecting the cells a decrease in staining for CD59 started to occur in both cell types. By 12 hours there was a homogeneous loss of CD59 staining in the HES cells. In Paju cells, on the other hand, CD59 was lost only from a proportion of the cells while most cells retained strong CD59 expression. The loss of CD59 from the HES cells resembled that seen in the inflamed epithelial cells in studies I and III.

With time the ability of the HES cells to control the C system weakened resulting in MAC deposition on the majority of the epithelial cells after exposure to serum. In contrast, the neuronal Paju cells mostly retained their C regulator expression and prevented MAC formation on the majority of the cells.

Human epithelial HES cells lost their ability to control C activation after they become infected with HSV-1. These cells could lose the GPI-anchored C regulators e.g. by membrane vesiculation, shedding or through the activity of the phospholipases that cleave the GPI-anchor[182,189]. Activation of the C system by the infected epithelial cells may be involved in the clearance of the infected cells. Compared to the epithelial cells, the neuronal cells are spared from C attack during the HSV-1 infection. A persistence of membrane C regulators and possible additional protective mechanisms could explain the limited susceptibility of neuronal cells to C attack. It has recently been suggested that HSV-1 infection can reactivate in oral mucosa in the absence of clinical symptoms. Furthermore, HSV-1-shedding could be detected also in recrudescence[190]. The results of this study support the possibility of limited viral replication without cellular destruction especially in neuronal cells.

The HSV-1 virus encodes the gC protein which has a well defined C regulatory activity[92]. This protein protects the maturing virions by inhibiting C lysis of the infected cells. The viral C control proteins inhibit lysis of most cells. As gC is mainly an alternative pathway inhibitor[191] the final extent of C regulation depends on the ability of the host to control the classical pathway. Cellular resistance thus depends on the combined activities of the viral and host cell C regulators. Noninfected cells of both types expressed the major cell-bound C regulators DAF, MCP and CD59. During the first hours of infection the expression of DAF and MCP intensified in both cell types whereas no up-regulation of the already strong expression of CD59 could be detected. However, we found that the contracted extensions of the Paju cells stained strongly positive for both CD59 and DAF. This probably is a result of up-regulation, re-arrangement and concentration of the proteins on the extensions. It is known that activated sprouting Paju cells up-regulate DAF expression on their surfaces[173]. DAF is thus the most dynamic membrane C regulator in its expression. Changes in the cellular environment can include signaling that leads to upregulation of DAF.

A recent study showed that an inherited C4 deficiency (together with HLA homozygosity) may predispose to frequent and unusually severe intraoral HSV-1 infections[93]. This suggests that a functional classical pathway of the C system is important in controlling HSV-1 infections. Future studies should explore whether deficiencies in the CP could predispose to more severe HSV-1 infections, like encephalitis. On the other hand, the present and earlier studies show that HSV-1-infected neuronal cells can control activation of the C system on their surfaces during the infection. However, the C system becomes activated and lytic MAC complexes are formed on one tenth of the infected neuronal cells. The activated C system could thus also be responsible for neuronal damage and malfunctions.

9 CONCLUSIONS

In general, an infection triggers an inflammatory response. The blood vessels in the infected area dilate and increased blood flow enables the anti-microbial mechanisms to rapidly reach the site and eliminate the invader. However, the inflammatory response also has many adverse effects which often cause more problems than the infection itself. The complement system has a central role in mediating inflammation and is thus responsible for a part of the adverse effects. The activation products C5a and C5b-9 activate white blood cells which in turn evoke a number of other inflammatory byproducts, including injurious cytokines, inflammatory enzymes, and cell adhesion molecules. Together, these byproducts can lead to the destruction of host tissue, as seen in many inflammatory diseases.

The present study has shown that the C system is active also in healthy gingival and gastric mucosa. It was found that the healthy mucosa is well protected against C activation and deposition of the terminal complexes is rare. However, the semipermeable barrier of the epithelial basement membrane (BM) is poorly protected against C activation and C deposits are systematically present. An infection enhances the activation and coarse deposits of C3 and C9 in the connective tissue can often be seen. The BM becomes disrupted and potentially leaky in many areas. It was also observed that the infection upregulated the production of the GPI-anchored inhibitors, DAF and CD59, while it also increased their instability. Granular staining for CD59 was typical for infected gingival and gastric mucosa and shedding of this protective protein into the gastric mucus was detected. Despite the shedding, MAC deposits were rare in the areas of CD59 expression. The BM and the underlying connective tissue were areas most susceptible to C damage.

Microbes have many mechanisms to evade complement-mediated killing. Identification of factors involved in complement resistance will be of much help in designing specific vaccines and new anti-microbial therapies. This study demonstrated a new mechanism whereby microbes can resist C-mediated lysis. It was found that *E. coli* and *H. pylori* bacteria may bind the GPI-anchored CD59 protein (protectin) released from host cell surfaces to their outer cell membranes. Binding of CD59 inhibits membrane attack complex assembly on the bacteria and may thereby contribute to their survival. The persistence of bound CD59 on the bacteria and the inability of the soluble CD59 to bind to the bacteria suggest that the GPI -lipid tail incorporates into the outer membrane analogously as to eukaryotic cell membranes (Fig. 4 and 7). This mechanism may partly explain the resistance of *H. pylori* against an active C system and the persistence of the chronic infection in the stomach. So far, it is the only suggested mechanism explaining how the normally C sensitive *H. pylori* could escape direct killing by C *in vivo*. It is probable that this mechanism functions mainly locally and under moderate C exposure and not *e.g.* in blood, which is a hostile environment for *H. pylori*.

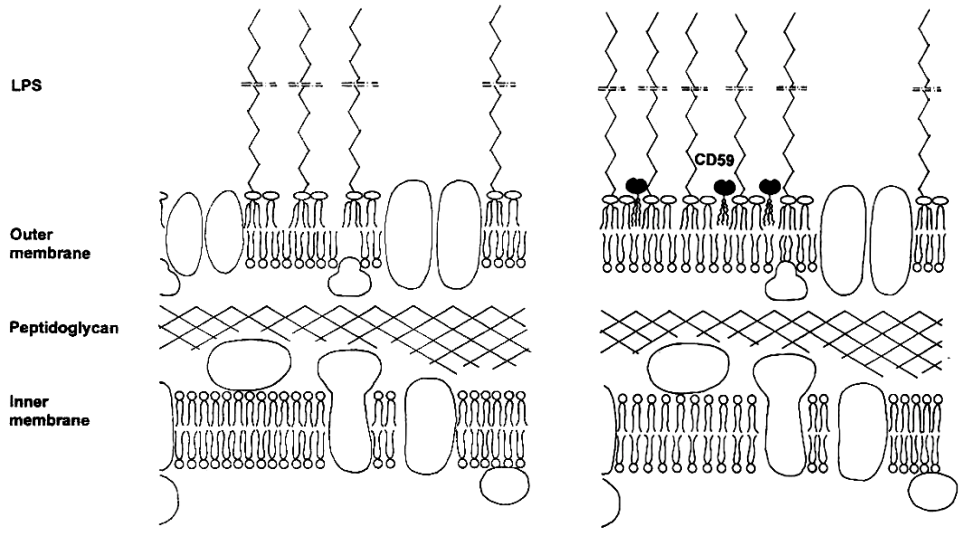

Figure 7. A schematic structure of the cell membrane of gram-negative bacteria (left) and a suggested binding site of human CD59 (right). The large, globular structures mark membrane proteins

As an example of the effects of a viral infection we studied the resistance of HSV-1-infected neuronal and epithelial cells to C activation *in vitro*. It was observed that both types of cells began to activate C after infection with HSV-1. Intact cells were well protected against C activation but an HSV-1 infection caused the epithelial cells to lose their protection. In the neuronal cells, however, the infection became latent and the expression of the membrane regulators of C persisted. HSV-1-infected neuronal cells were better than the epithelial cells in protecting themselves against C attack. Nevertheless, a small proportion of the infected neuronal cells deposited MAC on their surfaces suggesting their vulnerability to C attack, as well. It has recently been suggested that HSV-1 infection can reactivate in oral mucosa in the absence of clinical symptoms, and that HSV-1-shedding could be detected also in recrudescence[190]. The results of this study support the possibility of limited viral replication without cellular destruction, especially in neuronal cells.

In developing new therapies for mucosal infections it is important to understand their effects on the C system. Vaccinations and antibody treatments aim to strengthen the immune responses. However, additional C activation and excessive chemotaxin and anaphylatoxin production may excessively amplify inflammatory reactions both locally and systemically. On the other hand, treatments that enable specific control of certain C components could inhibit unnecessary tissue destruction. For example, the first medication of this type that will probably soon be in use is an anti-inflammatory antibody that selectively and potently binds to C5 and blocks the production of the inflammatory byproducts[192]. It inhibits inflammation dramatically but leaves the early steps of infection-preventing functions of C intact. A better understanding of the C system will enable the development of more sophisticated treatments for many infectious and inflammatory diseases.

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