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The role of gamma globulin, complement component 1q, factor B, properdin, body temperature, C-reactive protein and serum amyloid alpha to the activity and the function of the human complement system and its pathways

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ABSTRACT

The complement system plays a crucial role in orchestrating the activation and regulation of inflammation within the human immune system. Three distinct activation pathways—classical, lectin, and alternative—converge to form the common lytic pathway, culminating in the formation of the membrane-attacking complex that disrupts the structure of pathogens. Dysregulated complement system activity can lead to tissue damage, autoimmune diseases, or immune deficiencies.

In this study, the antimicrobial activity of human serum was investigated by using a bioluminescent microbe probe, *Escherichia coli* (pEGFPluxABCDEamp). This probe has previously been used to determine the antimicrobial activity of complement system and the polymorphonuclear neutrophils. In this study, blocking antibodies against key serum activators and components, including IgG, complement component 1q, factor B, and properdin, were utilized. The influence of body temperature and acute phase proteins, such as C reactive protein (CRP) and serum amyloid alpha (SAA), on the complement system was also examined.

The study reveals the critical factors influencing complement system activity and pathway function. Alongside crucial factors like C1q and IgG, alternative pathway components factor B and properdin played pivotal roles. Results indicated that the alternative pathway accounted for approximately one third of the overall serum antimicrobial activity, and blocking this pathway disrupted the entire complement system. Contrary to expectations, elevated body temperature during inflammation did not enhance the antimicrobial activity of human serum.

CRP demonstrated complement activation properties, but at higher physiological concentrations, it exhibited antagonistic tendencies, dampening the response. On the other hand, SAA enhanced the serum's activity.

Overall, this study sheds a light on the critical factors affecting both complement system activity and pathway functionality, emphasizing the importance of a balanced immune response.

1. Introduction

Complement system in blood and in interstitial fluid forms a backbone of the human immune system, activating and regulating the entire inflammatory machinery and participating in both humoral and cellular responses against fungi, bacteria, protozoa and viruses. Complement system acts as a general hub between the innate and adaptive immune system (Conigliaro et al., 2019; Friec and Kemper, 2009; Owen et al.,

Abbreviations: AP, Alternative Pathway; BL, Bioluminescence; C, Complement Component; CFU, Colony Forming Unit; CP, Classical Pathway; CRP, C-reactive Protein; CPM, Counts Per Minute; EGTA, Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid tetrasodium salt; fB, factor B; LP, Lectin Pathway; mAb, Monoclonal antibody; MBL, Mannose binding Lectin; OD, Optical Density; pAb, Polyclonal antibody; SAA, Serum Amyloid Alpha.

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2012; Seelen et al., 2005). After the recognition of the target structure, normally inactive components become sequentially activated in a cascade of proteolytic enzymes cleaving the next protein and simultaneously amplifying the reaction in every step (Conigliaro et al., 2019; Friec and Kemper, 2009; Owen et al., 2012; Seelen et al., 2005). Complement components (abbreviated here as C) carry out a sequence of events in recognition, activation and target lysis (Fig. 1). Complement system destroys the invading microbes by disrupting their membrane structure by forming the membrane attacking complexes (MAC) and by opsonizing targets to enhance phagocytosis. Soluble components like C3a and C5a are powerful chemotactic and anaphylactic agents regulating the whole immune response. Complement system is highly regulated because prolonged, disproportionate or mistimed activity can induce host tissue damage, autoimmune diseases or immune deficiency (Conigliaro et al., 2019; Vignesh et al., 2017). Complement affects many disease pathologies and environmental exposures, and is a key player in the inappropriate inflammatory activity in various infections like Covid-19 (Koelman et al., 2019; Makrides, 1998; Morgan and Meri, 1994; Noris and Remuzzi, 2013; Risitano et al., 2020). In the site of infection, complement components promote the clearance of microbial waste and apoptotic bodies by opsonizing them for phagocytosis (Conigliaro et al., 2019; Friec and Kemper, 2009; Noris and Remuzzi, 2013; Seelen et al., 2005).

Complement system consists of three separate activation pathways: classical (CP), alternative (AP) and lectin pathway (LP), illustrated in Fig. 1.

The analysis of the complement system in clinical immunology laboratory consists of the enzyme linked immunosorbent assays, turbidimetric methods assessing certain components and their reactions, and mass spectrometry-based analysis of the complement proteins (Kilpi et al., 2009; Ling and Murali, 2019; Vignesh et al., 2017). Functional methods are mainly end-point based, like the 50% complement hemolytic activity (CH50), assessing the whole serum capability to lyse sheep erythrocytes coated with anti-sheep antibodies and 50% alternative pathway (AP50) activity, analyzing the functionality of the alternative pathway based on the capacity of serum to lyse rabbit erythrocytes (Ling and Murali, 2019; Ohtani, 2020; Vignesh et al., 2017).

Molecular biology has enabled the construction of the recombinant reporter microbe probes providing a kinetical and real time-based method to detect the antimicrobial activity of the serum (Hakkila et al., 2002; Lehtinen et al., 2006; Virta et al., 1997). *Escherichia coli* K12, transformed with the bacterial luciferace gene cassette luxABCDE was utilized as a bioluminescent microbe probe in this study. Viable *E. coli* K-12 (pEGFPluxABCDEamp) is constitutively bioluminescent, providing a convenient way to measure viability and antimicrobial effects of the complement system in real-time basis (Atosuo et al., 2013).

E. coli-lux has been tested and compared with the microbiological reference methods like the colony forming unit plate counting (CFU) and the spectrophotometric optical density (OD) (Atosuo et al., 2013). The bioluminescence (BL) signal of the *E. coli*-lux suspension correlates with the number of living cells, whilst the decreasing BL signal indicates the number of dying *E. coli*-lux cells in the reaction (Atosuo et al., 2013; Kilpi et al., 2009; Virta et al., 1998).

The primary aim of this study was, by utilizing blocking antibodies against factor B (fB), C1q, IgG and properdin, to quantitate how CP, AP and LP responses together form the overall antimicrobial activity of the human serum (Fig. 1).

The second aim of this study was to ascertain how the body temperature and acute phase proteins like C-reactive protein (CRP) and serum amyloid A (SAA), the systemic signs and markers of inflammation/infection, affect the serum complement activity.

Increased body temperature is a spontaneous reaction to the inflammatory stimuli, and it is considered beneficial for the immune defense, broadly enhancing its activities (Blomqvist and Engblom, 2018). Theoretical human internal body temperature is 37 °C but it varies by the person (Dakappa, 2015). During inflammation, body temperature can increase even 5 °C degrees above normal. In this study, the activity of the complement system was tested between the range of 31 °C and

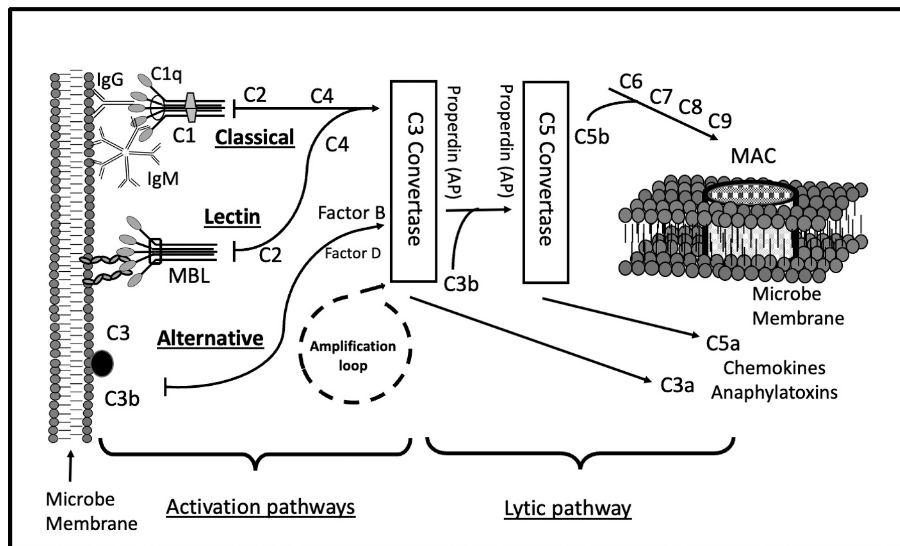

Fig. 1. Complement system consists of three separate activation pathways. The classical pathway (CP) is the part of the adaptive immune system since it is activated by the specific antibodies (IgG and/or IgM) attached to the exterior of the target. Antibodies are recognized by the C1 complex consisting of the one C1q, two C1r and two C1s subunits. The consecutive steps are the activation of C2 and C4 components and formation of CP-C3-convertase (C4bC2a) and in succession the C5 -convertase (C4bC2aC3b). The lectin Pathway is activated by the mannose binding lectin (MBL) attaching to a sugar residue like mannose on the microbe surface followed by the activation of C2 and C4 components which together with C3b constitute the C5-convertase like that of the CP (Noris and Remuzzi, 2013). The phylogenetically oldest is the alternative pathway (AP), previously called the properdin pathway (Harboe and Mollnes, 2008; Kouser et al., 2013). It occurs when the C3b is cleaved from C3 in spontaneous tic over reaction and attached to the pathogen structure. Amplification loop is a feedback system amplifying the signal from small trigger to a large downstream effect securing the C3b to the reaction (Lachmann, 2009). Pathogen-bound C3b binds with the factor-B, factor-D, and properdin when forming an AP-C3-convertase (C3bBbP) and consecutively C5 -convertase (C3bBbPC3b) (Noris and Remuzzi, 2013). All three activation pathways converge in C3-convertase phase comprising a common lytic pathway culminating to the formation of membrane attacking complex MAC. The CP activation is thought to be the most effective and even the main arm of the whole system (Conigliaro et al., 2019; Noris and Remuzzi, 2013).

43 °C.

According to the current knowledge, CRP and SAA are immune system activators. Both proteins are produced in the liver, and during the acute phase their levels increase considerably in blood (Gruys et al., 2005). CRP testing is commonly used as a non-specific bacterial infection marker. Subclinical CRP values are 10 mg/l or lower, but these values can frequently rise up to 200 mg/l in a patient with severe bacterial infection or septicemia. CRP is considered having a role as a complement activator through the classical pathway and C1q, but it is thought to also have a contradictory role in limiting the excessive inflammatory responses (Haapasalo and Meri, 2019; Willems et al., 2019; Zeller et al., 2021).

The role of SAA in the acute phase is not as well established as that of the CRP. SAA levels.

can rise as much as 1000-fold (from 1 to 1000 mg/l) in 24 h after acute phase response onset, and it participates in multiple roles during

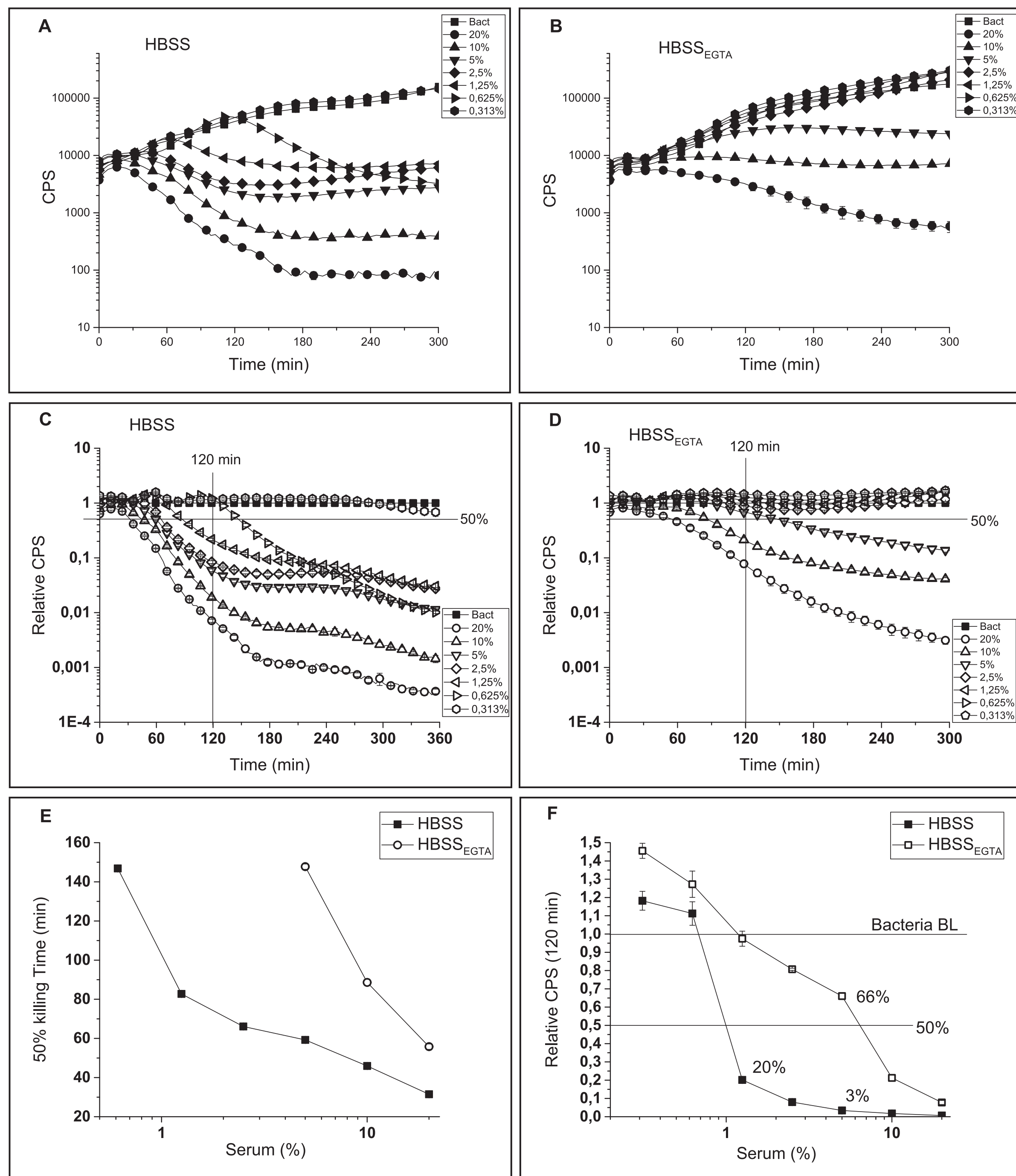

Fig. 2. The *E. coli*-lux kinetics in the various human whole serum (A) and EGTA serum (B) concentrations ranging from 0,313% to 20% and the corresponding relative CPS values in the whole serum (C) and in EGTA serum (D). The serum dose dependency of the time required to 50% CPS diminishment of the probe bacteria (E) and the serum dose relation to the CPS diminishment of the *E. coli*-lux probe after 120 min incubation (F). The upper vertical line represents the 100% viability of the bacterial suspension without the serum and lower the 50% viability/killing. The viability in 1,25% whole serum after 120 min of incubation was 20% (80% killing) and in 5% whole serum and EGTA serum 3% (97% killing) and 66% (34% killing) respectively. All results are mean values of the three parallel wells with standard deviation (±SD).

the inflammation, tissue damage or trauma (Sack Jr, 2018; Shimano et al., 2021; Sorić Hosman et al., 2021; Zhang et al., 2019). The role of SAA in the respect to the complement system is not fully known, and no diagnostic cut-off values have been established for the human SAA.

2. Results

2.1. Antimicrobial kinetics (Fig. 2)

The raw data of the antimicrobial whole serum (CP + AP + LP) and AP kinetics in various human serum concentrations are presented in Fig. 2A and B, and corresponding relative CPS values in Fig. 2C and D.

The whole serum, containing all three complement activation pathways, clearly had more efficient antimicrobial capacity than the AP alone (Fig. 2E and F). Only the two highest serum concentrations in AP (10% and 20%) were able to diminish the number of living bacteria below 50% during the first 120 min of incubation, while in the whole serum concentrations of ≥1,25% the 50% killing capacity was reached within the same time frame (Fig. 2F). In 5% serum, the AP killed 34% (leaving 66% intact) and the whole serum nearly all (97%, leaving 3% intact) of bacteria (Fig. 2F), indicating that the CP and the LP together account for about two-thirds of total antimicrobial capacity of serum complement system.

Low serum levels (0,313% and 0,625%) stimulated bacterial BL yield (Fig. 2F). This boosting effect is quite common when very small serum concentrations are used (data not shown).

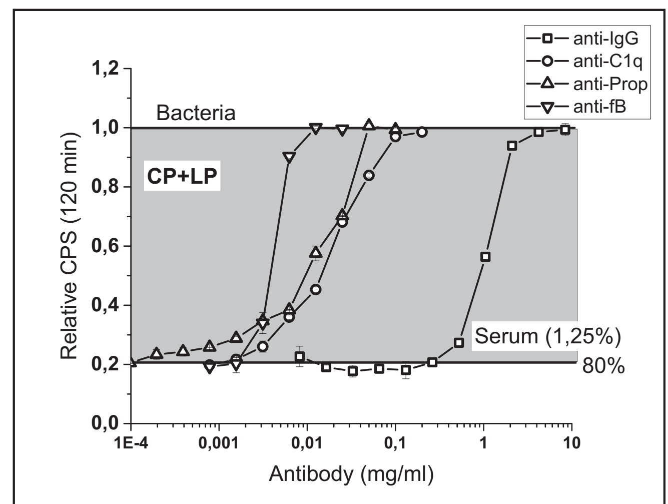

Fig. 4. The whole serum concentration of 1,25% killed 80% of the bacteria during the 120 min of incubation. The alternative pathway activity of the 1,25% serum only diminished 2,6% of the target *E.coli*-lux population. Curves represent the dose dependent effect of the blocking antibodies against IgG (from 0,0078 mg/ml to 2,0 mg/ml), C1q (from 0,00078 mg/ml to 0,2 mg/ml), prop (from 0,0001 mg/ml to 0,1 mg/ml) and fB (from 0,00078 mg/ml to 0,025 mg/ml) on the whole serum antimicrobial activity. All results are mean values of the three parallel wells with standard deviation (±SD).

2.2. The antibody blocking of the complement system (Figs. 3 and 4)

To test the hypothesis that AP is responsible for one third and CP + LP for two-thirds of total antimicrobial capacity of serum complement, we next utilized four different blocking antibodies against key components of AP (fB and properdin) and CP (C1q and IgG) in complement measurements.

When CP-blockers anti-C1q and anti-IgG were used in 5% serum (in HBSS), about two-thirds of the killing capacity was blocked, which is in line with the hypothesis (Fig. 3A and B). Surprisingly, potential AP-blockers anti-fB and anti-properdin blocked nearly all (85–90%) of the killing capacity of the serum, indicating that fB and properdin play a larger role in regulating the complement response than assumed. In 1,25% serum (in HBSS), where AP activity is low (Fig. 2C), the complement activity was blocked by all four antibodies (Fig. 4), further emphasizing the central role of fB and properdin in regulating complement system activity.

All those combinations of two antibodies where anti-fB, anti-properdin or both is/are present, blocked the killing capacity of the 5% serum entirely, while the combination of anti-C1q and anti-IgG blocked about two-thirds of the killing capacity of the serum (Fig. 3B).

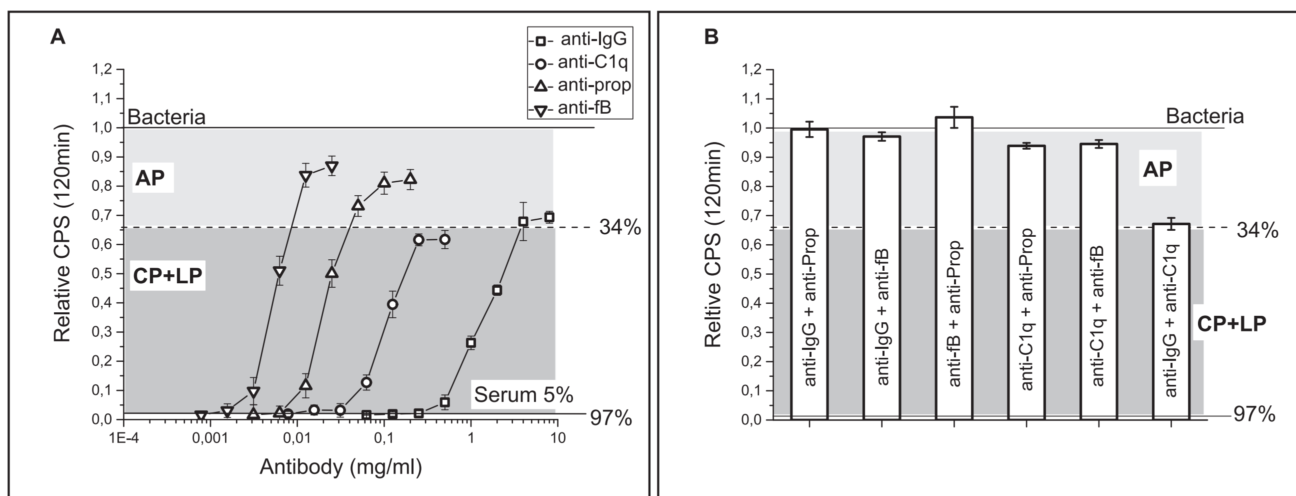

Fig. 3. (A) The whole serum concentration of 5% killed 97% of the bacteria during the 120 min of incubation. Vertical line and the light grey area represent the 34% portion of the alternative pathway activity of the 5% serum. Curves represent the dose dependent effect of the antibodies against IgG (from 0,0625 mg/ml to 8,0 mg/ml), C1q (from 0,00078 mg/ml to 0,5 mg/ml), Properdin (prop) (from 0,0031 mg/ml to 0,2 mg/ml) and Factor-B (fB) (from 0,00078 mg/ml to 0,025 mg/ml) on the whole serum antimicrobial activity.
(B) Columns represent the dose dependent effect of the antibody combinations with the highest concentrations (anti-IgG 8 mg/ml, anti-C1q 0,5 mg, anti-properdin 0,2 mg/ml and anti-fB 0,025 mg/ml) of each blocking antibody on the 5% whole serum antimicrobial activity. All results are mean values of the three parallel wells with standard deviation (±SD)

2.3. Temperature (Fig. 5)

At 37 °C, the 50% bacterial clearance time increased mildly at higher temperature in whole serum but stayed at relative constant level for AP responses. When going toward the lower temperatures, complement killing rates decreased progressively (Fig. 5).

2.4. CRP (Fig. 6A)

When 1.25% serum (in HBSS) was used, complement killing rates stayed constant at CRP levels ≤5 mg/l. A modest but clear enhancement of serum antimicrobial activity was observed at the CRP levels between 5 and 80 mg/l. Interestingly, at CRP levels >100 mg/l the killing rates decreased rapidly. CRP alone without serum did not promote any antimicrobial activity.

2.5. SAA (Fig. 6B)

After 120 min of incubation (Fig. 3C), it was noticed that SAA alone (without serum) had antimicrobial activity at SAA levels >8 mg/l and that 250 mg/ml of SAA killed about 65% of the bacteria. When 1.25% serum was present in the medium, the similar concentration dependent enhancement in bacterial killing by SAA was observed; 250 mg/l of SAA reduced the relative CPS response about 85% from 0.2 to 0.03.

3. Discussion

In the present study, it was clearly demonstrated that: 1) AP is responsible for one third and CP + LP for two-thirds of total antimicrobial capacity of the serum complement system against *E. coli*, 2) CP + LP response can be inhibited completely by CP blocking antibodies, anti-IgG and anti-C1q, leaving AP response still unaffected, 3) AP-blockers anti-fB and anti-properdin were needed in order to inhibit the complement system in its entirety.

From the results, the conclusions can be drawn that the unfunctional AP leads to unfunctional complement system since without the C3b component, required in all C5 convertase formation, the CP and LP are stagnated. It is known that both fB and especially properdin are important modifiers of the complement system activity by critically affecting the formation of C3-convertase, but they seem to have more crucial role that has been anticipated and the properdin pathway is a legitimate designation for the alternative pathway (Harboe and Mollnes, 2008; Kouser et al., 2013; Lachmann, 2009; Michels et al., 2019). The activation of the CP leads to an efficient antimicrobial effect, but the results emphasize the central role of AP and its components in the functional complement system. We can hypothesize that the primary role of the CP and its C3-convertase may be to produce a vast amount of covalently bound C3b opsonins on the surface of the invaded pathogen, and thus augment the activation of the AP. This would lead to the abundancy of the AP derived C5-convertase on the pathogen surfaces compared to that of the CP alone, but no such evidence has yet been shown, and the issue is the topic of the future investigations.

Complement system consists of the dozens of different essential components, and using the antibodies to block these to eliminate a certain function is not without a handicap. Firstly, most of the essential complement components are very abundant in blood and pathway blocking requires a vast number of antibodies. Secondly, the activation of the CP is based mainly on the recognition of the antibodies (IgG mainly) attached to the target surface, but also the antibody-antigen complexes can activate the CP and the AP in some extent (Noris and Remuzzi, 2013). The addition of the test antibody (normally IgG glass) to the reaction, in order to block the activation, may cause a formation of antigen-antibody complexes and thus presumably lead to the bias in the results of the assay e.g. the spontaneous activation of the cascade (Schifferli, 1996; Shmagel and Chereshnev, 2009). The role of serum proteins like albumin and other globulins, abundantly present in serum, is also an intriguing question.

What is the role of LP in antimicrobial activity? We were unable to find factors for this study that would have allowed us to differentiate the antimicrobial activity of CP and LP in a way that would enable us to analyze their function and activity separately. No functional antibodies were found. Theoretically, when C1 complex is blocked by anti-IgG and/or anti-C1q, the LP should still be active. However, according to the results presented, the role of LP in killing the *E. coli* seemed to be negligible. The Further studies with the functional anti-MBL (mannose-binding lectin) need to be done to clarify the issue.

Moreover, not only the blocking, but also the addition of certain activating components should be tested in future. The addition of purified human MBL could be one option which should hypothetically accelerate the antimicrobial activity of the LP.

Temperature is a critical factor in homeothermic animals like humans, and the homeostasis of this system is subjected under the rigorous regulation. However, during inflammatory reactions or trauma, body temperature is increased to assist in fighting against the possible intruders (Dakappa, 2015). According to results, we can conclude that fever (From 36 °C to 42 °C) only mildly increases the bacterial clearance in whole serum, at least not against the *E. coli*. Hypothermia, on the other hand, seems to prevent the autolytic activity of the complement system. This temperature increase during the fever reaction, did not affect to the antimicrobial activity of the AP. This could be advantageous during the surgeries operated at the hypothermic temperatures.

CRP and SAA are so called positive acute-phase proteins, meaning that their concentration in blood increases during the inflammation (Gruys et al., 2005). CRP acts as a complement system activator and as an opsonin, enhancing the phagocytosis. It has a dual role as an activator and as a suppressor of an excessive response (Haapasalo and Meri, 2019). This may be why, in high concentrations (>100 mg/l), CRP turns to be the complement system antagonist decreasing the activity as shown in this study (Fig. 6A). It is well established that CRP readings over 100 mg/l indicate severe inflammation, and the risk of septicemia elevates considerably. It is possible that in higher concentrations CRP possesses a retardant role, decelerating the overall inflammation response by reducing the activity of the complement system (Haapasalo and Meri, 2019). High concentrations of CRP can direct the response more toward the opsonization and phagocytosis. The antimicrobial effects of the complement system are bound to the formation of MAC causing the release of the anaphylatoxins on the way, and if this system is stagnated, the attached complement components can still act as an

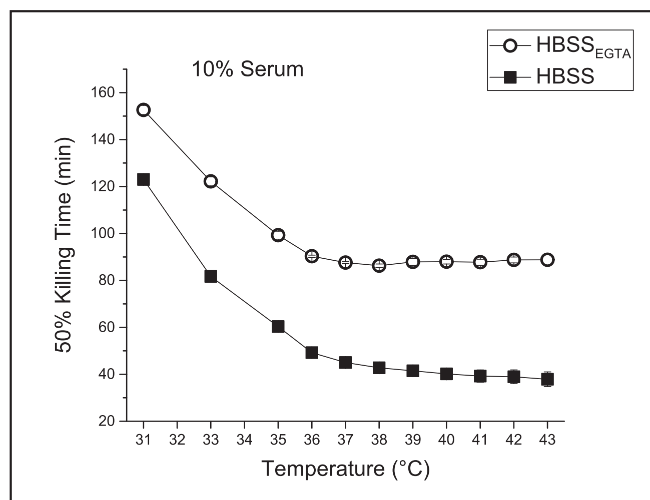

Fig. 5. The influence of the various temperatures (31 °C, 33 °C, 35 °C, 36 °C, 37 °C, 38 °C, 39 °C, 40 °C, 41 °C, 42 °C and 43 °C) on the time required to kill 50% of the *E. coli*-lux cells by 10% serum in HBSS and $HBSS_{EGTA}$. All results are mean values of the three parallel wells with standard deviation (±SD).

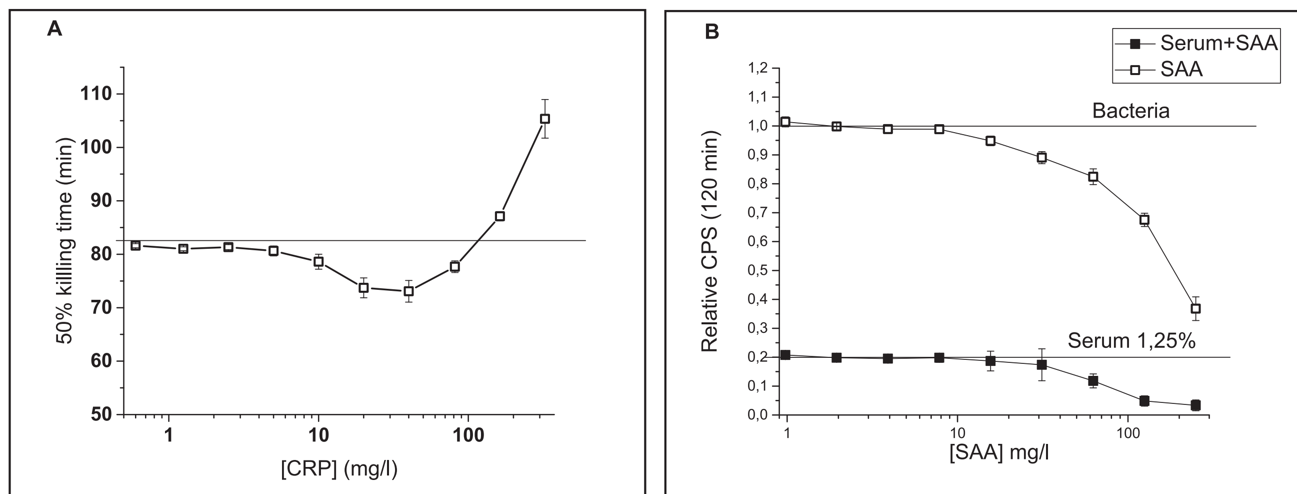

Fig. 6. (A) The influence of the various C-Reactive Protein concentrations (CRP) (320 mg/l, 160 mg/l, 80 mg/l, 40 mg/l, 20 mg/l, 10 mg/l, 5 mg/l, 2,5 mg/l, 1,25 mg/l, 0,6 mg/l, and 0 mg/l) on the time required to kill 50% of the probe *E. coli*-lux cells by 10% serum in HBSS at 37 °C.
(B) The influence of various human Serum Amyloid-A (SAA) concentrations (256 mg/l, 128 mg/l, 64 mg/l, 32 mg/l, 16 mg/l, 8 mg/l, 4 mg/l, 2 mg/l, 1 mg/l and 0 mg/l) on the probe bacteria *E. coli*-lux and on the whole serum (1,25%) antimicrobial activity. All results are mean values of the three parallel wells with standard deviation (±SD).

opsonizing factor facilitating the phagocytosis (Haapasalo and Meri, 2019).

The SAA is one of the many amyloids present in our body, participating in acute phase activities by providing anaphylactic duties. In this study it seems to facilitate the complement system activities and also promoted antimicrobial activities by itself in higher than 8 mg/l concentration (normal 1–10 mg/ml). During the acute phase, SAA values rapidly increase and it may prove to be an important factor in pathogen-host interactions, but further studies are needed.

The usage of the *E. coli*-lux as a probe enables the functional and real time-based method for the antimicrobial reactions in vitro (Atosuo et al., 2013; Suominen et al., 2020). The used recombinant *E. coli* -lux strain has particularly been engineered for the complement system research and there is previous research from human, bat and rainbow trout complement systems (Atosuo et al., 2013; Kilpi et al., 2009; Lilley et al., 2013; Vojtek et al., 2014).

In addition to complement system analysis, it has been utilized in the immune system research previously in assessment of the neutrophils' bacteria killing activities, and to reveal the antimicrobial roles of the myeloperoxidase and lactoperoxidase enzymes affecting in oxidative reactions connected to the neutrophil phagocytose functions (Atosuo et al., 2021; Atosuo and Suominen, 2019; Atosuo and Lilius, 2011; Schlorke et al., 2016). As a gram-negative commensal bacterium, the *E. coli* may offer a unilateral and simplified view of the actual events, since most severe pathogens are gram-positive species. Still, when these preconditions are acknowledged and kept in mind, *E. coli*-lux provides a handy model for the immunological ground research. However, the gram-positive strain microbe probe transferred with the lux-cassette could offer very fascinating insights to the antimicrobial activities alongside the *E. coli*-lux probe.

4. Materials and methods

4.1. Serum sample

The blood samples were collected from four healthy volunteers in 8 ml Vacuette serum gel-tubes (Greiner Bio-one, Kremsmünster, Austria). Serum was obtained by centrifugation at 1500 ×*g* for 10 min, after which individual serum samples were collected, pooled and stored in 50 μl aliquots at −80 °C in 2 ml Eppendorf tubes.

4.2. *Escherichia coli-lux preparation*

The *E. coli* K12 strain, transformed with the modified luciferase gene containing plasmid pEGFPluxABCDEamp, was used as a bacterial probe in complement system activity measurements (Atosuo et al., 2013). The expression of the whole operon produces the luciferase enzyme complex, resulting in *E. coli* cells emitting constitutive bioluminescence (BL) (Atosuo et al., 2013).This strain is designated hereafter as *E. coli*-lux. The BL signal of the bacteria is directly correlated with the viability of cells, and when the antimicrobial agent like human serum is added to the reaction, the BL signal diminishes in the accordance with the number of killed *E. coli*-lux cells.

E. coli-lux was cultivated in 100 ml Luria Bertani Broth (LBamp) (10 g tryptone [Neogen, Lansing, MI, USA], 5 g of yeast extract [Neogen, Lansing, MI, USA], 5 g NaCl [Sigma-Aldrich, St. Louis, Missouri, USA] and 100 μg/ml ampicillin [Sigma, St. Louis, Missouri, USA, pH 7.4] to maintain the selection pressure). The cultivation was incubated in a shaker (100 rpm) at 37 °C, until the bacteria suspension was at the end of its logarithmic growth phase at an optical density (OD) 0.450, defined by the photometric turbidity measurement at 620 nm (UV-1601 Shimadzu Spectrophotometer, Shimadzu Corp., Tokyo, Japan). Cells were washed and harvested by centrifugation (1500 ×*g*, 10 min), resuspended in 10 ml of LBamp containing 25% of glycerol, distributed in aliquots and kept frozen at −80 °C.

4.3. Complement activity assessment

Complement system activity was measured using microtiter plate wells (96-well plate, Greiner One, Düsseldorf, Germany). Hanks Balanced Salt Solution (HBSS) (Merck KGaA, Darmstadt, Germany) supplemented with 1 mg/ml of gelatin (Sigma-Aldrich, St. Louis, Missouri, USA) was used as a reaction buffer. For the alternative pathway assays, the serum was diluted in HBSS buffer supplemented with 10 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) (Sigma Aldrich), and was designated as $HBSS_{EGTA}$. EGTA removes Ca2+ from the reaction, blocking Ca2 + −dependent CP and LP while leaving Mg2 + −dependent alternative pathway (AP) intact (Atosuo et al., 2013; Kilpi et al., 2009).

The reaction was initiated by adding 25 μl of *E. coli*-lux dilution (1/2500 in HBSS or $HBSS_{EGTA}$) to the wells containing 75 μl of serum dilution in HBSS or $HBSS_{EGTA}$. The final reaction volume was 100 μl,

containing approximately 2 × 10^3 viable *E. coli*-lux cells (theoretical OD620nm = 0.0005) and 20%, 10%, 5%, 2,5%, 1,25%, 0,625%, 0,313% or 0% serum (Fig. 2). In experiments where CRP, SAA or blocking antibodies were involved, the 75 μl mixture of serum dilution and the tested reagent or the mixture of the reagents in HBSS was first preincubated at +4 °C for one hour. After incubation, the complement reaction was started normally by adding 25 μl *E. coli*-lux dilution to the wells.

BL kinetics were assessed by incubating the microtiter plate in the plate reader luminometer (Hidex Sense Plate Reader, Hidex, Turku, Finland) and by measuring the BL signal at 2-min intervals during the 300-min incubation period. Bioluminescence results were shown as the average CPS/well (counts per second/well) of three parallel wells counted (Fig. 2A and B).

4.4. Blocking antibodies

Blocking antibodies anti-fB (monoclonal Invitreogen, Waltham, MA, USA), anti-C1q (monoclonal (Invitreogen, Waltham, MA, USA), anti-IgG (Goat Anti-Human Polyclonal IgG, Fc Fragment specific, Lot:157705, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and anti-Properdin (Mouse Anti-Human monoclonal IgG, Lot: l1620, Santa Cruz Biotech, Dallas, TX, USA) were used to find out the roles of different complement activation pathways within the total complement activity of 1,25% and 5% serums.

Following antibody concentrations were used: 0,025, 0,0125, 0,00625, 0,00313, 0,00156, 0,00078 and 0 mg/ml for anti-fB, 0,5, 0,25, 0,125, 0,0625, 0,0313, 0,0156, 0,0078 and 0 mg/ml for anti-C1q, 8, 4, 2, 1, 0,5, 0,25, 0,125, 0,0625 and 0 mg/ml for anti-IgG and 0,2, 0,1, 0,05, 0,025, 0,0125, 0,00625, 0,00313, 0,00156, 0,00078, 0,00039, 0,0002, 0,0001 and 0 mg/ml for anti-Properdin. (Fig. 3A, B and 4).

When testing the effect of six possible combinations of two antibodies on complement activity of 5% serum, the highest concentrations of the anti-fB (0,025 mg/ml), anti-C1q (0,5 mg/ml), anti-IgG (8 mg/ml) and anti-Properdin (0,2 mg/ml) were used (Fig. 3B).

4.5. Temperature

To test the influence of the temperature on complement activity of 10% serum in HBSS and $HBSS_{EGTA}$, the BL measurement was performed at various temperatures (31 °C, 33 °C, 35 °C, 36 °C, 37 °C, 38 °C, 39 °C, 40 °C, 41 °C, 42 °C and 43 °C (Fig. 5).

4.6. CRP

In order to test the influence of human CRP (Millipore, Burlington, MA, USA) on complement activity of 1,25% serum, the following additional CRP concentrations were used: 320 mg/l, 160 mg/l, 80 mg/l, 40 mg/l, 20 mg/l, 10 mg/l, 5 mg/l, 2,5 mg/l, 1,25 mg/l, 0,6 mg/l, and 0 mg/l (Fig. 6A). Since the internal CRP concentration of the test serum determined with the rapid device QuicRead go (Orion, Turku, Finland) was 1 mg/l, the 1.25% serum had a CRP concentration of <0.01 mg/l before CRP supplementation.

4.7. SAA

When studying influence of human SAA (Abcam, Cambridge, UK) on complement activity of 1,25% serum, the following additional SAA concentrations were used: 256 mg/l, 128 mg/l, 64 mg/l, 32 mg/l, 16 mg/l, 8 mg/l, 4 mg/l, 2 mg/l, 1 mg/l and 0 mg/l (Fig. 6B).

4.8. Data analysis

Each reaction was performed in three parallel measurement wells, from which, averages and standard deviations were observed.

To make comparison between separate BL measurements easier, the kinetic raw data was converted to relative CPS values by dividing the serum values by the bacterial value without serum (thus relative bacterial CPS value is constant 1 at all time points) (Figs. 2C, D and F, 3A, B and C and 4C).

Two parameters, the time in which the relative BL (CPS) response of bacterial suspension has decreased by half (50% killing time) (horizontal line in Fig. 2C and D), and relative 120 min value (vertical line in Fig. 2C and D), were used to compare serum complement activities.

Raw data was compiled and analyzed with Excel, Version 2016 (Microsoft, Redmond, Washington, USA). Graphs were prepared with Origin, Version 2016 (Microcal, OriginLab, Massachusetts, USA).

4.9. Ethics

This study was performed according to the clinical standards of the Declaration of Helsinki. The study plan was approved by the ethical committee of the Turku University Central Hospital (Dnro: 59/1801/2019), and a written informed consent was obtained from every donor who participated.

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Author contributions

Doc. Janne Atosuo (JA): Corresponding author, main contributor to manuscript writing, and study conception.

MSc Outi Karhuvaara and **MSc Eetu Suominen**: Responsible for primary laboratory work and active participants in the writing process.

MSc Julia Virtanen: Contributed to laboratory work and data analysis.

PhD Liisa Vilén: Collected blood samples and participated in data analysis.

Professor Jari Nuutila: Provided the manuscript with immunological expertise.

These contributions highlight the diverse roles of each author in the research project and manuscript preparation.

CRediT authorship contribution statement

Janne Atosuo: Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Outi Karhuvaara:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Eetu Suominen:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Julia Virtanen:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Liisa Vilén:** Writing – original draft, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation. **Jari Nuutila:** Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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