Journal of Umm Al-Qura University for Medical Sciences

journal homepage: https://uqu.edu.sa/en/mj

Effect of Carcinoembryonic Antigen-related Cell Adhesion Molecule-binding Recombinant Polypeptide on the Killing of *Neisseria meningitidis* by Human Neutrophils

Abdel-Rahman Youssef [a, b, c *].

[a] *Department of Basic and Clinical Oral Sciences, Faculty of Dentistry, Umm Al-Qura University, Makkah, Saudi Arabia.*
[b] *Department of Microbiology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.*
[c] *School of Cellular & Molecular Medicine, University of Bristol, Bristol, UK.*

ARTICLE INFO

Article History:
Submission date: 3/5/2020
Accepted date: 17/12/2020

Keywords:
Neutrophils, Neisseria meningitidis, CEACAM1-binding recombinant polypeptide.

Conflict of Interest:
None to declare.

ABSTRACT

Background: Neutrophils are an essential part of innate immunity and play a crucial role in controlling infection caused by *Neisseria meningitidis*. The *Moraxella catarrhalis* ubiquitous surface protein A1 (UspA1)-based recombinant polypeptide binds to the carcinoembryonic antigen-related cellular adhesion molecule (CEACAM) 1 receptor on host cells and blocks binding of the mucosal pathogens to human epithelial cells and T cells.
Aim of the study: Since the CEACAM1 receptor is expressed on neutrophils, the aim of this study was to investigate the effect of CEACAM1-binding recombinant polypeptide on the ability of neutrophils to kill *Neisseria meningitidis*.
Methods: The effect of CEACAM1-binding recombinant polypeptide on the phagocytic killing of *Neisseria meningitidis* by neutrophils was assessed by incubation of neutrophils with CEACAM1-binding recombinant polypeptide (UspA1 $_{527–665}$) or with CEACAM1-non-binding polypeptide control (UspA1 $_{659–863}$) for one hour before infection with *Neisseria meningitidis*. The surviving bacteria were released and counted.
Results: 30 minutes after infection of neutrophils with *Neisseria meningitidis*, the survival of bacteria in presence of CEACAM1-binding recombinant polypeptide was 64% compared to 52% with control peptide and 43% without peptide. However, one-hour after infection, the surviving bacteria was 32% in presence of CEACAM1-binding recombinant polypeptide compared to 18% with control peptide and 22% without peptides.
Conclusion: Although CEACAM1-binding polypeptide reduced the killing of *Neisseria meningitidis* by neutrophils, it did not entirely stop phagocytosis and killing of bacteria.

1. Introduction

Neisseria meningitidis is an important cause of bacterial meningitis. The bacteria spread from person to person through exposure to respiratory secretion. Colonization of *Neisseria meningitidis* starts with adhesion to specific receptors on human mucosal epithelial cells. The colony opacity associated (Opa) proteins are outer membrane proteins of *Neisseria meningitidis* that mediate adherence to human cells. The carcinoembryonic antigen-related cellular adhesion molecule (CEACAM) family of receptors are the major receptors for Opa proteins [1,2].

Neutrophils are the most abundant type of leucocytes and are recruited to infected tissues. They play an essential role in inflammation and host protection against microbial infections [3]. Neutrophils are the first cells that arrive at the site of infection where they phagocytose and kill bacteria via oxidative and non-oxidative mechanisms [4,5]. In addition, neutrophils actively limit metastatic seeding by killing tumor cells [6,7].

During inflammation, CEACAM expression is enhanced and facilitates Opa interactions and consequently cellular attachment and invasion. The interaction of neutrophils with *Neisseria meningitidis* depends on the bacterial expression of Opa proteins, which allow neutrophils to effectively recognize, engulf and kill the pathogen [8,9]. The majority of Opa proteins bind to members of the CEACAM family [10]. Recognition by CEACAM depends on the presence of particular Opa protein variants [11]. The carcinoembryonic antigen family is involved in intercellular binding interactions that affect various normal and pathogenic processes associated with cellular growth and differentiation. Human neutrophils express four members of CEACAM family, CEACAM1, CEACAM8, CEACAM6, and CEACAM3 which are recognized by CD66a, CD66b, CD66c, and CD66d monoclonal antibodies respectively. CEACAM glycoproteins are surface adhesion molecules that play an important role in biological functions of neutrophils such as adhesion and phagocytosis [11–14]. Recent work indicated that CEACAM1-dependent intercellular binding confers survival signals that prevent neutrophil apoptosis, which might allow the persistence of these important phagocytes at a site of infection [15].

Several studies have shown that the *Moraxella catarrhalis* ubiquitous surface protein A1 (UspA1) has the ability to bind to a variety of human epithelial cell lines including A549 cells [16], Chang [17] and HEp-2 cells [18]. UspA1-cellular interactions occur through members of the CEACAM subfamily [16,19] which are targeted by several other respiratory bacteria including *Hemophilus influenzae and Neisseria meningitidis* [20,21]. UspA1-based recombinant polypeptide rD-7 represents amino acids 527-667 of UspA1. A linear recombinant polypeptide rD-7 corresponds to a region of the UspA1 stalk that possesses the configuration needed for receptor binding. It has been reported that rD-7 was able to inhibit the binding of several mucosal pathogens, including *Neisseria meningitidis*, to a range of CEACAM-expressing cell lines [19]. In addition, it has been demonstrated that rD-7 dramatically decreased the interaction of *Neisseria meningitidis* and *Hemophilus influenzae* with the human lung epithelial cell line A549 cells [19], and CD4 T cells [22] compared with CEACAM1-non-binding polypeptide control (rD-8Δ; UspA1 $_{659–863}$). Furthermore, rD-7 could act as a broadly effective anti-microbial agent by blocking adherence to CEACAM-expressing target cells and has the potential as a vaccine candidate against M. catarrhalis [23]. These findings suggest that rD-7 polypeptide has the potential to be used as an antimicrobial agent to prevent infection. If CEACAM1-binding recombinant polypeptide rD-7 can block adherence of bacteria to CEACAM-expressing cells, including neutrophils**,** it is not known if rD-7 polypeptide affects the ability of neutrophils to kill bacteria. The aim of this study was to explore the effect of CEACAM1-binding recombinant polypeptide rD-7 on the killing of bacteria by neutrophils.

2. Materials and Methods

This work was conducted in the laboratories of the School of Cellular and Molecular Medicine, University of Bristol, UK.

[*] **Corresponding Author**
Department of Basic and Clinical Oral Sciences, Faculty of Dentistry, Umm Al-Qura University, Makkah, Saudi Arabia.
E-mail address: amyoussef@uqu.edu.sa (*Abdel-Rahman Youssef*))
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2.1. Bacterial isolate

For studies on live bacteria, *Neisseria meningitidis* serogroup A strain C751 Opa-positive were grown on brain heart infusion (BHI) agar supplemented with 10% heated horse blood as described previously [24]. Bacteria were cultured at 37°C in a humidified atmosphere of 5% CO_2 for 16-20 hours.

2.2. Neutrophils isolation

Human blood was obtained from healthy volunteers in accordance with local guidelines. Sodium Citrate as an anticoagulant was added to whole blood at a ratio of 1:10. Neutrophils were isolated as described previously [25]. Briefly, 5 ml of blood was layered carefully over the Ficoll-Hypaque gradient separation media (Mono-Poly Resolving medium; ICN Biochemicals, Santa Ana, California) and centrifuged at 300 x g for 30 minutes in a swinging bucket rotor at room temperature. The neutrophils band was collected, washed, and resuspended in RPMI 1640 medium (Sigma).

2.3. Flow cytometry

The purity of isolated neutrophils was assessed by FITC labeled anti-CD66b monoclonal antibody using FACSCalibur flow cytometer (Becton-Dickinson). The results were analyzed by CellQuest (Becton-Dickinson).

2.4. Phagocytic killing assay

The phagocytic killing assays were done in polypropylene 2 ml tubes rotating end-over-end at 4 rpm at 37°C, as described by Virji & Heckels [8]. Before infection with *Neisseria meningitidis,* CEACAM - binding recombinant polypeptide rD-7 or rD-8Δ polypeptide control at 2µg/ml were added to neutrophils in RPMI 1640 medium and incubated for 60 minutes at 37°C in a humidified atmosphere of 5% CO_2. After that, neutrophils were infected with *Neisseria meningitidis* at a multiplicity of infection (MOI) of 1:1. Bacteria (2 x 10^6) were added to 2 x 10^6 neutrophils in a final volume of 400 µl of RPMI-1640 medium containing 1% fetal calf serum for 30 and 60 minutes at 37°C in a humidified atmosphere of 5% CO_2. At the end of the incubation time point (30 and 60 minutes), the samples were centrifuged at 100 x g for 5 minutes to separate the extracellular bacteria from the neutrophils. To evaluate the count of intracellular bacteria, the pellet was washed twice in phosphate-buffered saline (PBS), and then the bacteria were released with 1% saponin and plated on BHI agar. The bacteria were incubated at 37°C in a humidified atmosphere of 5% CO_2 overnight for viable counting the next day [26]. The percent of surviving bacteria was determined.

2.5. Colony immunoblotting of OpaD protein

Colony blots analysis of OpaD protein expressed by *Neisseria meningitides* were prepared as described previously [2]. *Neisseria meningitides* were incubated with neutrophils at MOI 1:1 for 60 minutes and surviving bacteria were released by 1% saponin. Overnight agar-grown colonies were lifted on nitrocellulose discs. The discs were air-dried, blocked in 5% milk in PBST (PBS with Tween) for 12 hours and incubated with anti-Opa antibody P514. The binding of antibody was detected using anti-mouse Ig/alkaline phosphatase conjugate. The blots were developed using BCIP (5-bromo-4-chloro-3-indolyl-phosphate) in conjunction with NBT (nitro blue tetrazolium) for detection of alkaline phosphatase activity (Sigma).

2.6. Statistical analysis

One-way analysis of variance (Graphpad Software Inc.) was applied to assess the CFU counts of ingested bacteria of the phagocytosis assays. P values of <0.05 were considered significant.

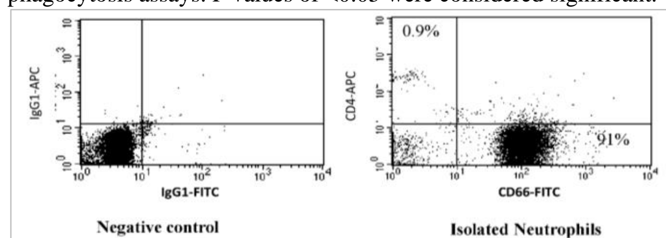


Figure 1. Flow cytometry analysis of isolated neutrophils. Neutrophils were labeled with FITC labeled anti-CD66b monoclonal antibody. CD66b positive neutrophils constitute 91% of the cell population.

3. Results

3.1. Isolation of neutrophils from peripheral blood

Neutrophils were isolated from peripheral blood and the purity of neutrophils collected was tested by flow cytometry. As can be seen in Fig.1, isolated neutrophils constitute 91% of the cell population.

3.2. Colony blot of OpaD protein

Colony blots analysis of OpaD protein expressed by *Neisseria meningitides* were prepared after incubation with neutrophils at MOI 1:1 The colony blot analysis (Fig. 2A) has shown that the inoculum had more than 99% Opa-positive and less than 1% Opa-negative bacteria. After 60 minutes of incubation with neutrophils, the number of Opa-negative revertant in the surviving population increased to about 5% (Fig. 2B).

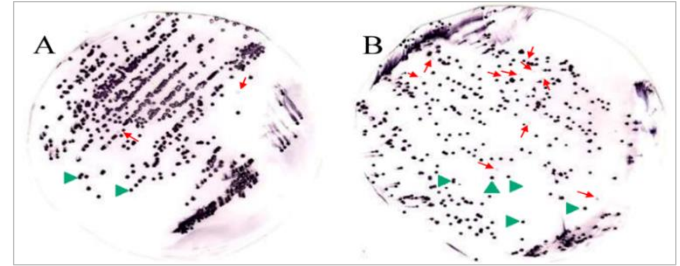
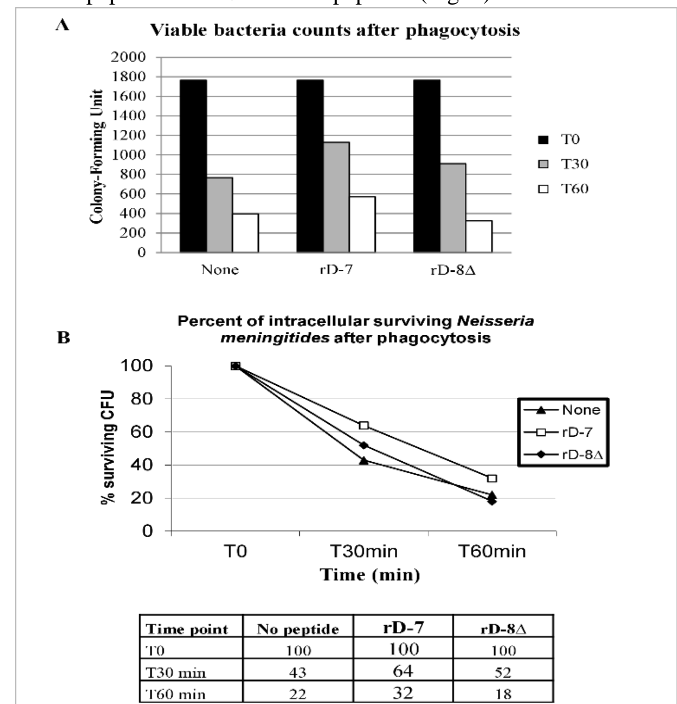


Figure 2. Colony blot analysis of Opa protein. *Neisseria meningitides* were incubated with neutrophils at MOI 1:1 for 60 minutes and surviving bacteria were released by saponin and plated on BHI agar. Colonies were lifted at time zero (A) and after 60 minutes (B) and Opa-positive colonies were identified with anti-Opa antibody. (A)The inoculum had more than 99% Opa-positive (green arrowhead) and less than 1% Opa-negative bacteria (red arrow). (B) After 60 minutes of incubation with neutrophils, the number of Opa-negative revertant in the surviving population increased to about 5%.

3.3. Effect of CEACAM1-binding recombinant polypeptide on phagocytosis

To assess the role of CEACAM1-binding recombinant polypeptide rD-7 on the phagocytic killing of *Neisseria meningitidis* by human neutrophils, the cells were incubated with CEACAM1-binding recombinant polypeptide rD-7 or CEACAM1-non-binding rD-8Δ polypeptide control for one hour. The neutrophils were then infected with *Neisseria meningitides* and the surviving bacteria were released and plated overnight. Colonies were counted (Fig. 3A) and the percentage was calculated (Fig. 3B) at each time point. Neutrophils killed bacteria significantly ($p < 0.0001$) after 30 and 60 minutes compared to T_0 (time zero). The survival of bacteria after 30 minutes incubation with neutrophils in presence of CEACAM1-binding recombinant polypeptide was 64% compared to 52% with control peptide and 43% without peptide. However, one hour after infection of neutrophils, the surviving bacteria was 32% in presence of CEACAM1-binding recombinant polypeptide compared to 18% in control peptide and 22% without peptides (Fig. 3).



Time point	No peptide	rD-7	rD-8Δ
T0	100	100	100
T30 min	43	64	52
T60 min	22	32	18

Figure 3. Effect of rD-7 on phagocytic killing of Opa+ *Neisseria meningitides* by human neutrophils. Neutrophils were incubated with CEACAM1-binding recombinant polypeptide rD-7 or CEACAM1-non-binding polypeptide rD-8Δ control for one hour and then infected with *Neisseria meningitides* and incubated for 30 and 60 minutes. The surviving bacteria were

released and plated overnight. Colonies were counted (Fig. 3A) and the percentage was calculated at each time points.

Therefore, the presence of CEACAM1-binding recombinant polypeptide rD-7 had reduced bacterial killing significantly ($p < 0.05$) compared with CEACAM1-non-binding polypeptide rD-8Δ control and without peptide.

4. Discussion.

In the present study, the purity of neutrophils was tested by flow cytometry which showed that 91% of the cell population was neutrophils. This result demonstrates that the isolation of neutrophils by Ficoll-Hypaque gradient separation media is effective for isolating a high yield of pure neutrophils. Also, the presence of Opa protein was confirmed by colony blot analysis.

During the process of bacterial infection, neutrophils migrate from blood to the site of infection where they phagocytose and kill bacteria [4,5]. In most cases of *Neisseria meningitidis* colonization, the innate immunity can stop the progress of the disease. It has been demonstrated that CEACAM1 present on human neutrophils can recognize Opa proteins of pathogenic Neisseria [2,10]. In addition, CEACAM1-binding recombinant polypeptide rD-7 was able to inhibit binding of *Neisseria meningitidis, Hemophilus influenzae* and *Moraxella catarrhalis* to the human cells that express CEACAM1 and CEACAM6 [19,22]. These studies suggest that rD-7 has the potential to be used as a vaccine from the important identification of the blocking effect of rD-7 on the phagocytic killing of phagocytes in the investigation.

The current study has demonstrated that CEACAM1-binding recombinant polypeptide rD-7 reduced the killing of *Neisseria meningitidis* by neutrophils, but it did not stop phagocytosis and killing of bacteria completely. To the best of my knowledge, this is the first study to examine the effect of rD-7 on the killing of *Neisseria meningitidis* by neutrophils. However, several studies have investigated the role of UspA1 and rD-7 in infection and inflammation [19,27–29].

It has been reported that the CEACAM1 binding motif of UspA proteins to be immunogenic and have adhesive blocking functions [19]. N'Guessan et al. [27] have shown that UspA1 protein of *M. catarrhalis* induces CEACAM1-dependent apoptosis in alveolar epithelial cells.

In addition, it has been demonstrated that monocyte differentiation was modulated by rD-7 and monocytes treated with rD-7 secreted high levels of IL-1ra (an inhibitor of the pro-inflammatory effects of IL-1α and IL-1β) and reduced the levels of pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 compared to lipopolysaccharide (LPS) [28]. These data suggest that rD-7 may regulate inflammation.

On the other hand, the specific engagement of CEACAM3 by UspA1 induces the activation of pro-inflammatory processes, such as neutrophils degranulation, reactive oxygen species (ROS) generation and chemokine release. The interaction of UspA1 with CEACAM3 induced the activation of the Nuclear factor κ B (NF-κB) pathway [29].

In this study, the number of surviving *Neisseria meningitidis* was reduced markedly after 60 minutes of incubation with neutrophils. However, pre-incubation of neutrophils with rD-7 CEACAM1-binding recombinant polypeptide but not rD-8Δ control reduced the killing of *Neisseria meningitidis* significantly. Therefore, the reduction in the killing power of bacteria by neutrophils may be due to the blocking effect of bacteria to CEACAM1-expressing neutrophils and consequently reducing phagocytosis and killing of these bacteria. On the other hand, phagocytosis was not inhibited completely by CEACAM binding polypeptide rD-7. This may be due to presence of many receptors for pathogen recognition on the surface of neutrophils. Some of these receptors are capable of innate recognition of microbial structures including; Toll-like receptors, C-type lectins, Nod-like receptors, and RIG-like receptors. Others are however, linked to the activation of the acquired immune response such as Fc-receptors [30]. Some phagocytic receptors can bind pathogen-associated molecular patterns (PAMPs) directly. Scavenger receptor (SR-A) can detect LPS on some gram-negative bacteria and on Neisseria meningitidis. Also, CD14 binds to LPS-binding protein and Mannose receptors bind mannan [31].

5. Conclusion

Although rD-7 polypeptide reduced the phagocytic killing of *Neisseria meningitidis* by neutrophils, it did not stop phagocytosis and the killing of these bacteria completely. Further investigations on the effect of rD-7 polypeptides on other immune cells are needed before using it as an antimicrobial agent to prevent bacterial colonization.

6. Acknowledgment

I am gratefully acknowledging the provision of r-D7 polypeptides by Professor Mumtaz Virji (University of Bristol, UK) for generously providing the recombinant polypeptides.

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