Cooperation between lysozyme and complement system in bactericidal action of human serum – is everything already clear?

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Abstract

The complement system plays an important role in protection of higher organisms against bacterial infection. Lysozyme cooperates with the complement system in the bactericidal action of serum. Adsorption of serum onto bentonite (montmorillonite, MMT) is well known procedure to remove the lysozyme from serum. Our present results show that the most efficient killing of Salmonella O48 occurred when all components of normal human serum (NHS) cooperated with each other. It is very interesting that elimination of lysozyme from NHS by using MMT significantly decreased the bactericidal activity of NHS against Salmonella O48 strain. The results of X-ray diffractometric studies suggested that apart from lysozyme, other components of serum were adsorbed on the bentonite particles.

Key words: *lysozyme, montmorillonite, normal human serum, Salmonella.*

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Introduction

Complement (C) plays a fundamental role in mediating and enhancing humoral immunity [1, 2]. The major role of the C system is to recognize and promote clearance of invading microorganisms. Deposition of C3b or C4b components of C onto the surface of a microorganism and the insertion of the membrane attack complex (MAC) into its cell membrane disrupts the integrity of the cell lipid membrane bilayer and killing the microorganism by osmotic lysis [1, 2].

Lysozyme is the enzyme that catalyses the hydrolysis of β 1,4 linkage between *N*-acetyloglucosamine and *N*-acetylmuramic acid in the bacterial cell wall. This enzyme lyses mostly Gram-positive and a few Gram-negative bacteria [3]. Donaldson et al. [4] has shown that the most efficient killing of Gram-negative bacteria by serum occurred when β-lysin, lysozyme and the C system acted together at usual serum concentrations.

The genus *Salmonella* as a member of the *Enterobacteriaceae* family, it is most known as an agent causing diarrhoeal disease in humans. *S. enterica* subspecies *enterica* are mainly associated with warm-blooded vertebrates and are usually transmitted by food or water contaminated by infected faeces [5, 6]. *Salmonella enterica* subsp. *enterica* is one of the main causative agents of food-borne disease in man, and can also be the cause of serious systemic illness [7]. Bacteria from *Salmonella* O48 serotype are clinically important strains causing intestinal disfunctions and diarrhoea [8]. All strains from this serotype contain the sialic acid (*N*-acetylneuraminic acid, NeuAc) inside the lipopolysaccharide (LPS) molecule [9]. Sialic acid as a component of outer membranes of bacteria, may play an important role in protecting the bacterial cells against the lytic activity of C. NeuAc bound on the bacterial surface has been shown to prevent activation of alternative pathway by increasing the affinity of H for C3b bound to the surface [10].

Our previous experimental results [11] indicated that strains *Salmonella* O48 with sialic acid – containing LPS demonstrate different sensitivity to the bactericidal action of normal human serum (NHS). The scope of this work is a comparison of the susceptibility to the NHS devoid of

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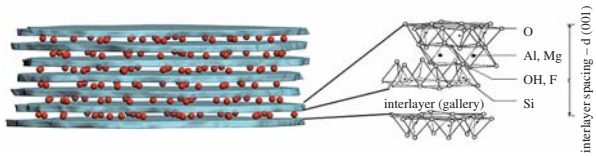

Fig. 1. The structure of montmorillonite (MMT)

lysozyme of some *Salmonella* serovar's belonging to O48 serotype. The aim of structural research was to trace transitions accompanying adsorption of proteins on the particles of montmorillonite.

Montmorillonite is a layered silicate derived from naturally occurring clay mineral called bentonite. It is composed of 1-nm thick crystalline layers separated by a gap that usually is referred to as gallery (Fig. 1). One of the most important properties of this material is ability adsorb molecules from liquid environments by strong ionic (ion-exchange occurring within galleries) and dispersive interactions. It is known that some of proteins adsorb strongly and readily on mineral surfaces. Amongst others, lysozyme is one of the proteins being 'jailed' on the surface of the silicate or carbon nanoparticles during the adsorption process [12-14].

Materials and Methods

Bacterial strains

The study was carried out on 7 strains of serotype *Salmonella* O48 which containing sialic acid in the O-specific side chain of LPS. The complete list of tested strains is presented in Table 1.

Serum

Normal Human Serum (NHS) was obtained from five donors who have not suffered from evident infections *Salmonella*. The people were not previously treated with antibiotics. The samples of serum were collected, pulled and kept frozen (–70°C) for a period no longer than three months. The suitable volume of serum was thawed immediately before using. Each portion was used only once.

Bentonite (montmorillonite, MMT)

We used the natural sodium montmorillonite extracting and purifying industrial (Górniczo-Metalowe Zakłady Zębiec, Poland).

Determination of the level of C3 and C4

The level of C3 and C4 components in NHS was determined using the specific antibodies.

The nutrient agar plates were used with monospecific polyclonal antibodies (MEGA – TRADIN – Gliwice, Poland) anti-C3 and anti-C4 proteins. The assay was carried out according to the instructions provided by the supplier.

Bentonite (montmorillonite, MMT) – adsorbed serum

Lysozyme removal from NHS was accomplished by 20 min absorption with 10 mg of washed MMT per ml at 4°C. MMT was washed three times in physiological saline (10 mg/ml), every time centrifuged at 4000 rpm for 20 min. at 4°C and then mixed with NHS (10 mg/ml) at 37°C for 10 min. The mixture was then centrifuged at 4000 rpm for 10 min. at 20°C and the supernatant serum was removed [4, 15]. This MMT – adsorbed serum was used in the experiments concerning the bactericidal action of NHS.

Afterwards palletized samples were used in SAXS and WAXS measurements.

Bactericidal activity of normal human serum (NHS)

The bactericidal activity of NHS was determined as described previously [16]. Briefly, the strains were grown overnight, and then bacterial cells in early exponential growth phase were transferred to fresh YP medium and incubated at 37°C for 1 hour in water bath. After incubation the bacterial cells were centrifuged (4000 rpm for 20 min.

Table 1. The origin and antigenic characteristics of *Salmonella* O48 strains used in this study

Species	Subspecies	Serovar	Antygen O48	Source	
Salmonella enterica	*enterica*	Dahlem	k:e:n:z_{15}	PCM 2512	KOS 1166
		Djakarta	z_4,z_{24}:-	PCM 2513	KOS 432
		Hisingen	a:1,5,7	PCM 2536	NBIMCC 1357
		Toucra	z:1,5,/z_{58}/	PCM 2515	KOS 1386
		Sydney	1:z	PCM 2551	IP 38/65
		Isaszeg	z_{10}:e,n,x	PCM 2550	IP 886/71
		Fitzroy	e,h:1,5	PCM 2549	IP 407/68

IP – Institute Pasteur, Paris, France; KOS – National Salmonella Centre, Gdansk, Poland; NBIMCC – National Bank of Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria; PCM – Polish Collection of Microorganisms, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland

at 4°C) and suspended in saline. Then the bacteria were mixed with 50% NHS (the serum was diluted with 0.1M NaCl). Bacteria with serum were incubated in a water bath at 37°C. After 0, 60 and 180 min, samples were collected, diluted, and cultured on nutrient agar plates for 18 h at 37°C. The number of colony forming units (CFU) at time 0 was taken as 100%. Strains with >100% survival in 50% serum after 180 min. of incubation were considered resistant. Strains with <100% survival were considered susceptible.

Thermal inactivation of NHS [16]

NHS decomplemented by heating the sample at 56°C for 30 min (NHS 56°C) was used as the control.

Structural investigations-adsorption of the proteins on montmorillonite [17]

Wide- and small angle X-ray diffraction

WAXS (wide angle X-ray scattering) experiments were performed on a SEIFERT URD6 diffractometer with Ni-filtered CuKα radiation generated by sealed X-ray tube. The radiation source was powered by a high-voltage generator operated at 40 kV and 30 mA. Data was collected in a step-scan mode (in 0,1°/2θ steps) within the range of 2θ from 1° to 15° that gives s-vector ranging from 0.11 nm^{-1} to 2.25 nm^{-1} ($s=2\sin\theta/\lambda$).

SAXS (small angle X-ray scattering) measurements were done using an evacuated Hecus-M'Braun Kratky camera with slit collimation system and linear, 1024 channel, position-sensitive detector. The instrument was equipped with a copper anode X-ray tube powered by Philips PW-1830 generator set at 30 kV and 10 mA. SAXS patterns were recorded for 15 minutes within s range of 0.015 and 0.9 nm^{-1}.

Results

The level of C3 in NHS was found to be 117.14 mg/dL (standard for NHS: 55-120 mg/dL) and the level of C4 was found to be 25.67 mg/dL (standard for NHS: 20-50 mg/dL).

All *Salmonella enterica* O48 strains investigated by us were susceptible to the bactericidal action of NHS. These results were presented at 6th European Congress of Chemotherapy and Infection and 24th Reunion Interdiscipilinaire de Chimiotherapie Anti-Infectieuse in Paris in 2004 [11].

Our present obtained results concerning the sensitivity of *Salmonella* O48 strains to NHS-MMT (serum treated with bentonite – MMT) are given in Tables 2. One serum concentrations (50%) were used and analysed strains showed variable sensitivity to the bactericidal action of NHS-MMT. Two serovars of *Salmonella* O48 (Hisingen PCM 2536 and Dahlem PCM 2512) were sensitive to the bactericidal effect of human complement in which the lysozyme was removed. Five strains (Toucra PCM 2515, Fitzroy PCM 2549, Isaszeg PCM 2550, Djakarta PCM 2513 and Sydney PCM 2551) demonstrated higher resistance to the bactericidal activity of NHS-MMT. After serum treated with MMT, the ratio of percentage survival of bacterial cells was above 1122.4 to 2526.3% after 3 hour of incubation. In those cases the bactericidal activity of NHS was lost after the MMT treatment. The results presented in Table 2 indicated that lysozyme was necessary in bactericidal action of normal human serum.

When NHS was heated at 56°C for 30 min all bactericidal activity of NHS was removed.

Table 2. Bactericidal activity of NHS-MMT (serum treated with bentonite – MMT, to remove lysozyme)

Salmonella O48 strain No.	50% NHS-MMT				50% NHS	
	CFU[1]				% of survival of bacterial cells in serum after 3 h of incubation	
	T0[2]	T1 (after 60 min)	T3 (after 180 min)	% of survival of bacterial cells in serum after 3 h of incubation	Control I[3]	Control II[4]
Hisingen PCM 2536	33×10^5	66×10^4	14×10^5	42.4	0.1	789.5
Dahlem PCM 2512	23×10^5	21×10^5	17×10^5	73.9	0.004	3863.6
Toucra PCM 2515	49×10^5	89×10^5	55×10^6	1122.4	0.8	3540.2
Fitzroy PCM 2549	18×10^5	12×10^6	31×10^6	1722.2	0.006	3250.0
Isaszeg PCM 2550	21×10^5	63×10^5	38×10^6	1809.5	0.03	4285.7
Djakarta PCM 2513	31×10^5	13×10^6	61×10^6	1967.7	0.005	3631.6
Sydney PCM 2551	19×10^5	73×10^5	48×10^6	2526.3	0.7	3722.0

[1] *CFU – colony forming units;* [2] *T0 – 100% of survival of bacterial cells in serum;* [3] *Control I – % of survival of bacterial cells in NHS after 3 h of incubation;*
[4] *Control II – % of survival of bacterial cells in NHS decomplemented by heating at 56°C for 30 min.*

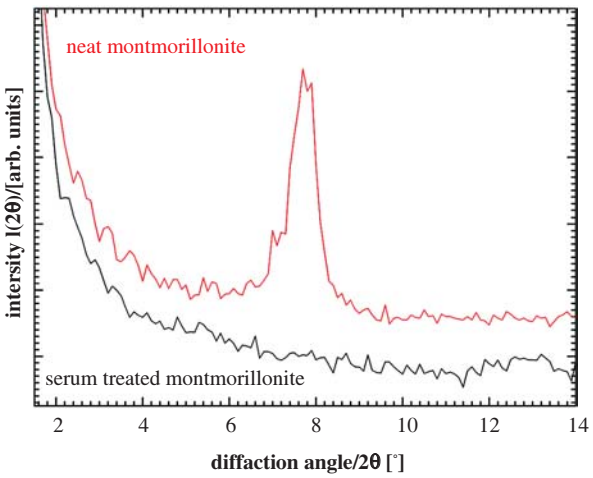

Fig. 2. WAXS traces recorded for unmodified montmorillonite (upper/red/curve) and the montmorillonite treated with serum (lower/black/curve)

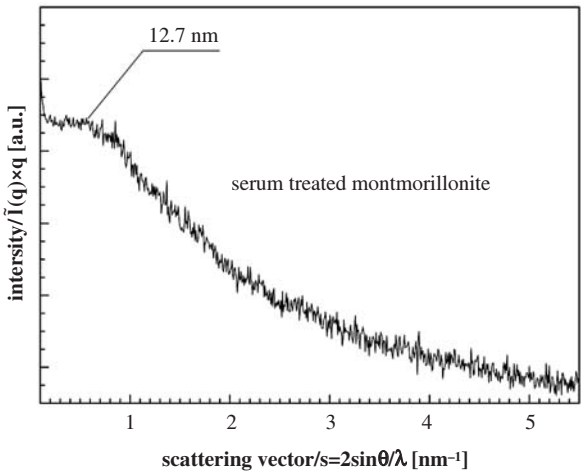

Fig. 3. One dimensional, smeared, Lorentz corrected SAXS pattern recorded for montmorillonite treated with serum.

X-ray diffractometric studies

A wide-angle X-ray diffractogram (WAXS) recorded for neat montmorillonite within 2θ of 1-15° (Fig. 2, upper curve) reveals a discrete maximum at diffraction angle (2θ) of approximately 8°, which indicates the existence of regular, layered structure of the intact mineral. The interlayer distance that can calculated according to the Braggs' law (1) from this maximum equaled 1.1 nm.

$$d = \frac{n\lambda}{2\sin\theta_{max}} \quad (1)$$

where:

d – interlayer distance
n – diffraction order (here is always equal to unity)
λ – wavelength of the X-ray beam (here CuK_α=0,1542 nm)
θ_{max} – the location of diffraction maximum expressed as half of diffraction angle (2θ)

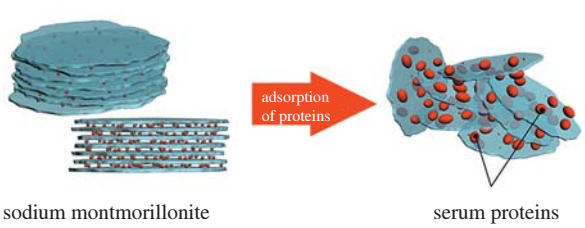

Fig. 4. Cartoon representation of structural changes in crystal structure of montmorillonite occurring upon adsorption of proteins from NHS

On the contrary, the WAXS curve recorded for the silicate with adsorbed proteins is featureless (Fig. 3, lower curve), which proves that stratified arrangement of the layered crystal after adsorption no longer exists. Analysis of the intensity scattered in low angle region recorded by means of small-angle X-ray scattering technique (SAXS) reveals that there was no long range ordering of silicate platelets at higher length scales. Although in the SAXS curve recorded for montmorillonite treated with serum (Fig. 3) one can see a broad and indistinct quasi-maximum located at s=0.08 nm^{-1}, its analysis based on Fourier transformation indicates that this peak does not originate from stacked platelets of the silicate. Thus, one can conclude that montmorillonite exfoliates during the adsorption, forming disordered system of statistically oriented silicate platelets according to the simplified model shown in Fig. 4.

Discussion

The role of lysozyme in the bactericidal action of serum is controversial. Many authors [18, 19], using sera from which lysozyme has been removed came to a conclusion that the lysozyme was unnecessary in the bacterial process. Other workers reported that the sera from which lysozyme was removed have a weaker bactericidal activity against various Gram-negative rods, as compared to that of NHS [20, 21].

In our experiment we proved that lysozyme participates as an obligatory factor in the bactericidal action of the components of normal human serum. The reduction the bactericidal activity of NHS after MMT treatment is very interesting. Adsorption of serum onto MMT is well known procedure to remove the lysozyme from serum. The easiest way to investigate mechanism of the adsorption is monitoring the value of interlayer spacing (d(001)) by means

of X-ray diffraction techniques. On the basis of the recorded values and structural behavior, one can estimate the dimensions of molecules adsorbed in the interlayer regions of the silicate and interactions between the molecules and nanoparticles [12-14].

In order to find which protein causes the observed change of the structure, MMT was treated with solutions containing two important components of the serum i.e. albumin and lysozyme. Also, for the sake of comparison adsorption from a solution containing the mixture of these proteins was investigated. Then the SAXS experiments were repeated according to normal procedures. As concluded from SAXS curves, in these cases no exfoliation was observed. This indicates that the structure change is caused by yet-not--identified protein or by synergistic adsorption of several components of the serum. Since, according to literature [22] adsorption of single proteins of polypeptides does not cause exfoliation, while the second was observed, we assumed that the mechanism of exfoliation relies on synergistic adsorption of several proteins on surface of the silicate. Aramini et al. [23] suggested that lysozyme such as some milk protein, citrates and phosphates is know to have high affinity binding sites for calcium. Calcium ions are required for classical/lectin pathway complement activation, and lysozyme absorbed onto MMT may act as inhibitor of this activation for strains which required this pathway complement activation. Hill et al. [24] suggested that the β-lysin and C1q may be removed (adsorbed on MMT) together with lysozyme. It is possible that onto MMT is removing also an unidentified factor essential in activation of complement. Currently this phenomenon is being investigated at our laboratories.

Conclusion

1. Adsorption of serum onto bentonite (MMT) is well known procedure to remove the lysozyme from serum.
2. It is very interesting that elimination of lysozyme from NHS by using MMT significantly decreased the bactericidal activity of NHS.
3. The results of X-ray diffractometric studies suggested that apart from lysozyme, other components of serum were adsorbed on the MMT particles.
4. It is suggested that the β-lysin and C1q may be removed (adsorbed on MMT) together with lysozyme.

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