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A COMPARATIVE STUDY OF THE HEMOLYTIC PROPERTIES OF SOME MICROORGANISMS

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In a previous investigation it was established that the plague hemolysin is a substance with characteristics of higher fatty acid (Tkachenko and Domaradskiy, present symposium).

At a concluding stage of investigation of the nature of hemolytic activity of the plague bacterium we were faced with the task to clarify how much species-specific is the ascertained nature of the plague hemolysin. With a view to this we devoted the present investigation to a comparative study of hemolytic properties of some other microbes: Staphylococcus aureus, Bacillus anthracis, Escherichia coli, and the causative agent of pseudotuberculosis in rodents.

One of the numerous hemolysins of Staph. aureus "nonspecific δ-hemolysin," described by Marx and Vaughan (1950), by its properties very much resembles a higher fatty acid, if we exclude its insolubility in acetone (Van Heyningen, 1953).

The hemolytic properties of B. anthracis are apparently connected not only with a C-lecithinase produced by it (Williams, 1957; Costlow, 1958; and others). It is known that this microbe has also a thermostabile hemolysin which by its thermostability resembles the plague hemolysin studied by us.

According to the data of a number of authors (Sonnenschein, 1930, and others, quoted after V. D. Shtiben and I. K.

Babich, 1955) the hemolytic properties in E. coli are connected with the action of bacteriophage upon it. We have earlier already noted the increase of hemolytic properties in plague cultures upon their experimental infection with a bacteriophage (Tkachenko, 1961, unpublished data). The study of hemolytic activity of Bact. pseudotuberculosis is of obvious interest in connection with well-known difficulties in differentiation of Bact. pestis from Bact. pseudotuberculosis.

METHODOLOGY

For a comparative study of the hemolytic activity were selected the following microbes: a strain of B. anthracis (vaccine STI), a strain of Staph. aureus, a strain of E. coli and seven strains of Bact. pseudotuberculosis, preserved at the Museum of the Live Cultures of the Irkutsk Antiplague Institute*.

In the experiments were used 48-hour cultures of the above-enumerated strains, grown in Hottinger's agar (pH 7.2) at 37° (pseudotuberculous cultures were grown at 28°, and in individual cases at 37°).

The investigation for hemolytic activity was carried out according to a technique used for the study of plague hemolysins -- first -- by growing cultures in the media with blood, then by testing "resting" cells and the obtained from them lyophilized preparations and different extracts, as it was explained in detail in previous works (Tkachenko, 1961, unpublished data; Tkachenko, 1961a; Tkachenko and Krotova, 1962, present symposium; Tkachenko and Domaradskiy, present symposium).

The evaluation of hemolytic activity of cultures of the microbes in question, under conditions of growing at 37° in Hottinger's media (pH 7.2), was performed with regard to the onset of hemolysis. Whereupon were estimated: the magnitude of hemolytic zones in the blood agar and the height of the column of broth stained with hemoglobin of lysed unwashed erythrocytes of the guinea pig.

The quantitative evaluation of hemolytic activity of washed-off microbial cultures and different preparations obtained from them was effected according to the technique developed by V. V. Tkachenko (1961b) and based on the estimate of 50%-hemolysis, using standardized suspensions of washed-off erythrocytes of the guinea pig, and in some experiments --

*The strain of B. anthracis STI was obtained from the Irkutsk Institute of Microbiology and Epidemiology.

other animals and men. In this case, the terminal concentrations of washed-off microbial cultures and their preparations in the experiments corresponded to those obtained during testing of the plague bacterium (Tkachenko, 1961a; Tkachenko and Krotova, present symposium, Tkachenko and Domaradskiy, present symposium).

RESULTS OF EXPERIMENTS

A comparative study of the hemolytic activity of Staph. aureus, B. anthracis STI, E. coli, and Bact. pseudotuberculosis permitted the clarification of some of its peculiarities as compared with the activity of plague bacterium (vaccine strains 1, 17 EB). According to the data obtained by us (Table 1) not a single from among washed-off and aerated microbial cultures tested, except, plague cultures, was capable to produce hemolysis.

The lyophilized washed-off cultures of Staph. aureus, B. anthracis and E. coli, at least during two-year observation of them remained hemolytically inactive. In connection with this we found it rational to pass at once to the preparation of lipids of lyophilized cultures of the aforesaid microbes and to test them for hemolytic activity.

The free and combined lipids of Staph. aureus were found to be devoid of hemolytic properties. According to Vaszi and Farcas (1961), the lipids of Staph. aureus contain unsaturated fatty acids in meager amounts, while the unsaturated fatty acids as compared with the saturated ones have more pronounced hemolytic properties (Greisman, 1958, 1959). Apparently, a nonspecific δ-hemolysin with characteristics of higher fatty acid, described by Marx and Vaughan, is hardly a product of destruction of a bacterial cell similarly to the plague hemolysin studied by us. Manifestly, δ-hemolysin is only a product of the splitting of lipids present in the culture medium of Staph. aureus and a consequence of lipolytic activity of the latter. This assumption fully agrees with investigational data of G. N. Chistovich (1961) on the accumulation of hemolytically active higher fatty acids in the culture medium of Staph. aureus as a result of the hydrolysis of lipids, catalyzed by Staphylococcal lecithovitellinase.

Free and combined lipids of B. anthracis STI and E. coli produced the lysis of washed-off erythrocytes. The yield of free lipids of B. anthracis STI was comparatively high (up to 7-8%, with the yield of lipids of investigated gram-negative bacteria within the limits of 2-3%), and their emulsifiability in physiological solution approximately corresponded to that observed in lipids of Bact. pestis, but in hemolytic activity

TABLE 1

Hemolytic Activity of Various Microbial Cultures and of Preparations Obtained from Them

Вид микроба (1)	Гемолитическая активность (3)								
	в отношении неотмытых эритроцитов морской свинки (4)	в отношении отмытых эритроцитов морской свинки (5)							
		при суспендировании в физрастворе (7)				при эмульгировании в физрастворе (8)			
		до лиофилизации (9)		после лиофилизации (10)					
	при выращивании на средах Хоттингера (pH 7,2) (6)	неотмытых бактерий (11)	отмытых и аэрированных бактерий (12)	неотмытых бактерий (11)	отмытых аэрированных бактерий (12)	водонерастворимых остатков отмытых аэрированных бактерий (13)	свободных липидов (14)		связанных липидов (15)	
							исходных (16)	ацетонорастворимых фракций (17)	исходных (16)	ацетонорастворимых фракций (17)
Bac. anthracis СТИ (2)	слабая и непостоянная (18)	—	—	—	—	не испытывался (19)	умеренно выраженная и постоянная (20)	сильнее выраженная и постоянная (21)	умеренно выраженная и непостоянная (22)	не испытывался (23)
Bact. coli	—	—	—	—	—		резко выраженная и постоянная	резко выраженная и постоянная	резко выраженная и непостоянная	резко выраженная и непостоянная

TABLE 1 (Cont'd)

Вид микроба (1)	Гемолитическая активность (3)									
	в отношении неотмытых эритроцитов морской свинки (4)	в отношении отмытых эритроцитов морской свинки (5)								
		при суспендировании в физрастворе (7)					при эмульгировании в физрастворе (8)			
		до лиофилизации (9)		после лиофилизации (10)			свободных липидов (14)		связанных липидов (15)	
	при выращивании на средах Хоттингера (pH 7,2) (6)	неотмытых бактерий (11)	отмытых и аэрированных бактерий (12)	неотмытых бактерий (11)	отмытых аэрированных бактерий (12)	водонерастворимых остатков отмытых аэрированных бактерий (13)	исходных (16)	ацетонорастворимых фракций (17)	исходных (18)	ацетонорастворимых фракций (19)
Bact. pestis	слабая и непостоянная (13)	слабая и непостоянная (13)	слабая и непостоянная (13)	умеренно выраженная и постоянная (20)	умеренно выраженная и постоянная (20)	сильно выраженная и постоянная (23)	резко выраженная и постоянная (22)	резко выраженная и постоянная (22)	резко выраженная и постоянная (22)	резко выраженная и постоянная (22)
Bact. pseudotuberculosis rodentium	·	—	—	слабо выраженная и постоянная (24)	слабо выраженная и постоянная (24)	·	·	·	·	·
Staphylococcus aureus	(22) резко выраженная и постоянная	—	—	—	—	не испытывался (22)	—	—	—	не испытывался (22)

1 - Species of microbes; 2 - STI; 3 - Hemolytic activity; 4 - with respect to unwashed erythrocytes of guinea pig; 5 - with respect to washed-off erythrocytes of guinea pig; 6 - when grown in Hottinger media (pH 7.2); 7 - after suspension in physiological solution; 8 - after emulsification in physiological solution; 9 - before lyophilization; 10 - after lyophilization; 11 -- unwashed-off bacteria;

[key continued]

[key to Table 1 continued]

12 - washed-off and aerated bacteria; 13 - water-insoluble residues of washed-off and aerated bacteria; 14 - free lipids; 15 - combined lipids; 16 - original; 17 - acetone-soluble fractions; 18 - weak and unstable; 19 - not tested; 20 - moderately pronounced and stable; 21 - more strongly pronounced and stable; 22 - sharply pronounced and stable; 23 - strongly pronounced and stable; 24 - weakly pronounced and stable.

they were greatly inferior to the latter. The lipids of E. coli were less well emulsified in physiological solution but as to degree of hemolytic activity resembled the lipids of Bact. pestis. Upon fractionation with acetone the hemolytic activity of the lipids of E. coli, as in lipids of Bact. pestis, wholly passed to acetone soluble fraction.

Very interesting data were obtained from the hemolytic activity of Bact. pseudotuberculosis. The latter, like Bact. pestis, when grown in the media with defibrinated blood, produced a weak and unstable hemolysis, whereupon erythrocytes of the sheep and horse were lysed less well than erythrocytes of the guinea pig and rabbit; human erythrocytes were not lysed at all; the lysis of dog's erythrocytes was unstable and most pronounced. However, the unwashed-off aerated pseudotuberculous cultures, suspended in physiological solution, lost the capacity to produce lysis of washed-off erythrocytes and other animals. Lyophilized pseudotuberculous cultures, like plague cultures during storage acquired hemolytic properties, although after comparatively long periods of time: within three-four months up to one year after lyophilization. And what is more similarly to the plague bacterium, the water insoluble residue of pseudotuberculous bacterium obtained according to the methods of Baker et al. and Walker-Domaradskiy, were found to be also hemolytically active, whereas the water-soluble, mainly protein fractions and lipopolysaccharide, obtained by Davies' method, were devoid of hemolytic properties. We shall note that since lipopolysaccharide of the pseudotuberculous bacterium is considered as its toxin (Davies, 1958), we may assume that hemolytic activity of pseudotuberculous bacterium is not connected with its toxin.

The lyophilized pseudotuberculous bacteria and their water-insoluble residues were inferior to the corresponding preparations of washed-off aerated plague bacteria, as to degree of hemolytic activity. Nevertheless in a great number of characteristics (thermostability, activity of erythrocytes of different species, inhibiting action of protein, ions of calcium and magnesium, etc.) the hemolytic properties of lyophilized cultures of pseudotuberculous and plague bacteria and their water insoluble residues were found to be identical.

TABLE 2

Capacity of Lipids of E. coli, Bact. pseudotuberculosis and Bact. pestis to Lyse Washed-off Erythrocytes of Various Animals and Men (incubation at 37°)

Отмытые эритроциты (1)	Наступление 50%-ного гемолиза, вызываемого липидами, в минутах (7)											
	кишечной палочки (8)				псевдотуберкулезного микроба* (9)				чумного микроба (10)			
	свободные липиды (11)		связанные липиды (12)		свободные липиды (11)		связанные липиды (12)		свободные липиды (11)		связанные липиды (12)	
	исходные (13)	ацетонорастворимая фракция (14)	исходные (13)	ацетонорастворимая фракция (14)	исходные (13)	ацетонорастворимая фракция (14)	исходные (13)	ацетонорастворимая фракция (14)	исходные (13)	ацетонорастворимая фракция (14)	исходные (13)	ацетонорастворимая фракция (14)
(2) (I) морской свинки . .	16,0	17,0	18,2	16,3	12,6	11,5	11,5	10,6	14,2	14,2	14,2	13,5
(3) (II) лошади	21 0	22,5	2 ,1	21,4	18,3	17,4	17,0	16,7	18,5	19,0	20,0	20,0
(4) (III) кролика	29,0	28,3	29,3	27,5	23,5	23,0	22,7	22,5	26,0	24,6	25,3	25,0
(5) (IV) человека	31,3	33,6	31,3	32,1	29,5	28,1	27,4	27,0	31,0	29,0	30,0	28,5
(6) V) барана	61,6	60,1	57,2	60,0	55,8	51,2	55,0	53,6	57,6	56,1	51,5	55,0

*According to data obtained in testing lipids extracted from lyophilized culture of Bact. pseudotuberculosis rodentium 1.

1 - Washed-off erythrocytes; 2 - Guinea pig; 3 - Horse; 4 - Rabbit; 5 -- Men; 6 - Sheep; 7 - Onset of 50%-hemolysis caused by lipids in minutes; 8 - E. coli; 9 - Bact. pseudotuberculosis; 10 - Bact. pestis; 11 - Free lipids; 12 - Combined lipids; 13 - Original; 14 -- Acetone-soluble fraction.

TABLE 3

Variations of Hemolytic Activity of the Lipids of E. coli, Bact. pseudotuberculosis and Bact. pestis Depending on the Influence pH of the Medium, Ions of Calcium and Magnesium, and Cholesterol (Washed-off erythrocytes incubated at 37°)

Среда	Наступление 50%-ного гемолиза, вызванного липидами, в минутах											
	кишечной палочки				псевдотуберкулезного микроба				чумного микроба			
	свободные липиды		связанные липиды		свободные липиды		связанные липиды		свободные липиды		связанные липиды	
	исходные	ацетонорастворимая фракция	исходные	ацетонорастворимая фракция	исходные	ацетонорастворимая фракция	исходные	ацетонорастворимая фракция	исходные	ацетонорастворимая фракция	исходные	ацетонорастворимая фракция
Физраствор + 0,6 м-фосфатный буфер pH 5,71	26,0	26,3	28,0	25,8	21,0	19,3	19,4	18,3	19,3	21,0	19,5	21,0
pH 7,23	не испыт.	не испыт.	не испыт.	не испыт.	10,0	9,6	9,7	9,4	13,1	12,0	12,2	11,0
pH 7,82	10,2	10,0	12,5	9,5	6,8	6,1	6,3	5,9	10,5	5,8	8,2	5,5
Физраствор (контроль) pH 6,92—7,31	16,5	16,8	17,0	16,3	11,6	11,5	11,7	11,1	15,0	14,2	14,0	14,0
Физраствор + $3,5 \cdot 10^{-3}$ м Ca	43,5	45,1	45,6	46,7	62,9	60,2	62,3	59,1	72,0	72,5	69,8	70,0
+ $3,25 \cdot 10^{-3}$ м Mg	42,0	41	не испыт.	не испыт.	53,6	52,3	51,7	51,5	57,0	55,0	56,0	54,5
+ 0,29% холестерина	41,5	41,5	43,0	40,5	38,9	36,9	36,9	37,1	41,8	39,8	39,5	38,2
Физраствор (контроль)	16,0	17,2	17,6	16,3	12,5	11,7	11,8	10,8	14,5	14,5	14,0	13,5

1 - Medium; 2 - Physiological solution +0.6 M-phosphate buffer pH 5.71; 3 - Physiological solution (control); 4 - Physiological solution...; 5 - ...cholesterol; 6 - Onset of 50%-hemolysis caused by lipids, in minutes; 7 - E. coli; 8 - Bact. pseudotuberculosis; 9 - Bact. pestis; 10 - free lipids; 11 - combined lipids; 12 - original; 13 - acetone-soluble fraction.

The similarity of hemolysins of different bacteria is enhanced by the fact that they are extracted from lyophilized cells as free and combined lipids, and during fractionation of the latter with the aid of acetone it is fully revealed in the acetone-soluble fraction*. The hemolytic activity of lipids of pseudotuberculous bacterium and of their acetone-soluble fractions exhibits the same characteristics which were ascertained in the [illegible] lyophilized bacterial cells and in their water-insoluble residues (Tables 2 and 3). The emulsifiability of lipids of Bact. pseudotuberculosis was found to be comparatively high.

Thus, we have been able to ascertain the hemolytic properties of lipids, not only in Bact. pestis but also in some other microbes: E. coli, B. anthracis and Bact. pseudotuberculosis. The ascertained hemolytic activity of the above microbes is in many respects similar to that studied earlier in Bact. pestis (Tkachenko and Domaradskiy, present symposium), and by its nature is apparently also due to the presence of higher fatty acids contained in these microbes.

The hemolytic properties of acetone-soluble fraction of different hemolytically active lipids of bacterial origin, including lipids of plague bacterium, were compared in parallel experiments with hemolytic properties of the higher fatty acids: of saturated (stearic acid) and unsaturated(oleic acid) series**. Hemolytic activity of the oleate and stearate, similarly to the activity of lipids of E. coli, Bact. pestis and Bact. pseudotuberculosis manifested itself in regard to erythrocytes of different species displayed high thermostability, was inhibited by protein, ions of calcium and magnesium and excess of hydrogen ions, etc. However, as to the degree of hemolytic activity both acids were inferior to bacterial lipids, whereupon the stearate was hemolytically less active than oleate.

Thus, the determination of hemolytic activity of the lipids of Bact. pseudotuberculosis, E. coli and B. anthracis convincingly showed that the presence of hemolytic properties in the lipids of Bact. pestis is not a species-specific characteristic.

*The lipids were extracted from lyophilized cultures of two strains of Bact. pseudotuberculosis.

**In the present experiments there was deliberately formed an elevated concentration of higher fatty acids as compared with that in bacterial lipids (10%-emulsion in physiological solution). Emulsiability of both acids was enhanced by using their sodium salts in experiments.

The similarity of hemolytic properties of the lipids of Bact. pestis and Bact. pseudotuberculosis is enhanced by the fact that in the starting lyophilized cultures of these microbes and their water-insoluble residues the hemolytic properties are analogous, although in pseudotuberculous cultures their manifestation is delayed (during storage) and they are less pronounced.

CONCLUSIONS

1. The lipids of Bact. pestis, Bact. pseudotuberculosis, B. anthracis STI and E. coli, as well as acetone-soluble fractions of these lipids are capable of causing lysis of washed-off erythrocytes of the guinea pig. The lipids of Staph. aureus are devoid of hemolytic properties.

2. The starting lyophilized cultures of the aforesaid microbes, except Bact. pestis and Bact. pseudotuberculosis are hemolytically inactive. The hemolytic properties of lyophilized pseudotuberculous bacteria manifest themselves after a more or less prolonged time after lyophilization. The water-insoluble residues of these bacteria from the very beginning display comparatively well-pronounced hemolytic properties, just as the water-insoluble residue of Bact. pestis.

3. The hemolytic activity of all bacterial lipids tested ~~by us, in regard to a great number of properties~~ is similar to the hemolytic activity of oleic and stearic acids. This confirms our assumption that plague hemolysin and hemolysins of other microbes tested, ~~by us~~, concentrated in lipids of the latter, are apparently higher fatty acids, mainly of the saturated series.

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