THE HEMOLYTIC PROPERTIES OF THE LIPIDES OF THE PLAGUE BACILLUS

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[Following is a translation of an article by V. V. Tkachenko and I. V. Domaradskiy in the Russian-language periodical Izvestiya Irkutskogo gosudarstvennogo nauchnoissledovatel'skogo protivochumnogo instituta Sibiri i Dal'nego Vostoka (News of the Irkutsk State Scientific Research Anti-Plague Institute of Siberia and the Far East), Vol 25, 1963, pages 129-134.]

Through preliminary tests it was established that the treating of lyophilized plague bacteria and their water insoluble residues with organic solvents leads to a sharp lowering of their hemolytic activity. The most pronounced lowering of the hemolytic activity occurred upon treating these preparations with ether, acetone, and n-butanol; treating with ethanol produced the least expressed lowering of the hemolytic activity. In this case the water insoluble residues of the plague microbe lost their hemolytic properties rather easily; the extracts consisting primarily of free lipides displayed sharply expressed hemolytic properties. These observations led us to obtain lipides of the plague microbe in order to study their hemolytic activity.

Method

Two-day cultures of the plague microbe (strain EB) cultivated in aerated Hottinger's broth (pH 7.2) at 28 degrees Centigrade were washed with a physiological solution of ordinary salt, were lyophilized and maintained over calcined calcium chloride in a vacuum desiccator.

The free lipides were extracted by treating the dry bacteria with ether in a Soxhlet apparatus; the combined lipides were obtained using the Bogdanov method (Belozerskiy and Proskuryakova, 1951). Then the residues of the bacteria were once again treated with ether.

The free and combined lipides were subjected to fractioning with acetone. For the fullest removal of the phospholipides the acetone extracts of free and combined lipides after the removal of the acetone were again treated with dry acetone cooled to -10 degrees (Grossman, 1959).

In order to achieve the fullest removal of moisture and organic solvents all the preparations of free and combined lipides together with their acetone-soluble and acetone-insoluble fractions were kept in a vacuum desiccator over calcined calcium chloride and pieces of paraffin.

A quantitative calculation of the hemolytic activity of all the preparations which were obtained was conducted in accordance with the method developed by V. V. Tkachenko (1961a, 1961b) using standardized suspensions of washed erythrocytes of the guinea pig and in some tests of other animals and man. The final concentration of each of the lipide preparations being investigated in the test was equal to 0.015 mg/ml. The initial lyophilized plague bacteria which were tested by us at the same time were used in the same final concentrations as in the preceding investigation -- 5 mg/ml per sample (Tkachenko and Krotova, this collection).

Results of Tests

The free and combined lipides of the plague microbe and also their acetone-soluble fractions turned out to be hemolytically active. The acetone-insoluble fractions of lipides of the plague microbe consisting primarily of phospholipides lacked hemolytic properties.

Consequently, the hemolytically active factor of the plague microbe cannot be the phospholipide, since being soluble in acetone it passes wholly into the acetone-soluble fraction as both free and combined lipides. This circumstance very graphically reveals the mechanism of the lowering of the hemolytic activity of lyophilized cultures of the plague microbe and their water-insoluble fractions, as was noted by us upon drying with acetone (Tkachenko and Krotova, this collection).

The yield of lipides and their acetone-soluble fractions from lyophilized plague bacteria is given in Table 1.

Table 1

Yield of Lipides of the Plague Microbe in Percent

Free lipides*		Combined lipides		
Initial	Acetone-soluble fraction	Initial	Acetone-soluble fraction	Total lipides
2.16	1.58	2.04	1.45	4.2

Remarks: 1. The acetone-soluble fraction in the case of free lipides comprised on the average 73% of the latter and 71% in the case of combined lipides.

2. The table contains average values of three determinations of the yield of one or the other lipide of lyophilized cultures of the plague microbe of various series of cultivation.

* According to the data of V. I. Bystrenin (1940), the yield of free lipides is 3.24-4.2%.

In comparing the hemolytic activity of lipides and whole bacteria cells of the plague microbe it was found that lipides and especially their acetone-soluble fractions possess considerably higher hemolytic activity (Table 2).

Table 2

Hemolytic Activity of Lipides of the Plague Microbe and their Acetone-soluble Fractions

Preparations of lipides of the plague microbe	Hemolytic activity in arbitrary units (based on 0.001 mg of preparation)
Free lipides	9,137
Acetone-soluble fraction of free lipides	12,045
Combined lipides	9,040
Acetone-soluble fraction of combined lipides	14,527

From Table 2 it is evident that combined lipides of the plague microbe and their acetone-soluble fraction are hemolytically more active than free lipides of the plague microbe and their acetone-soluble fraction.

Thus in these tests we obtained and tested highly concentrated preparations of plague hemolysin.

There is a direct relation between the concentration of lipide preparations of the plague microbe and the time of occurrence of 50% lysis of washed erythrocytes of the guinea pig caused by these preparations. The minimum hemolytic dose which is able to cause 50% hemolysis under conditions of incubation at 37 degrees for one hour for lipide preparations lies in the range of 0.001 mg/ml.

After preliminary 3-hour boiling (with a reverse condenser) of 0.1% emulsions of lipides of the plague microbe and their acetone soluble fractions in a physical solution their hemolytic activity not only was not lowered but actually increased somewhat (by 1.2-1.3 times).

Lipides of the plague microbe and their acetone-soluble fractions retained the ability to cause hemolysis even at a temperature of 4 degrees Centigrade, although the minimum hemolytic dose increased in this case by 33-36 times.

Lipide preparations of the plague microbe lyse erythrocytes of the same species of animals and of man as do the initial lyophilized cultures and their water-insoluble residues; with respect to their degree of sensitivity to lytic action of lipides the erythrocytes are arranged in the previous order (Table 3).

Table 3

Hemolytic Activity of Lipides of the Plague Microbe with Respect to Erythrocytes of Various Animals and Man

	Hemolytic activity in arbitrary units (based on 0.001 mg of preparation)			
	Free lipides		Combined lipides	
Erythrocytes of a:	Initial	Acetone-soluble fraction	Initial	Acetone-soluble fraction
Guinea pig	9,445	12,583	9,809	14,805
Horse	7,276	9,539	6,980	10,009
Rabbit	5,177	7,367	5,506	8,023
Man	4,338	6,234	4,953	7,033
Ram	2,344	3,211	2,730	3,649

In this case as in the case of the erythrocytes of the guinea pig the hemolytic activity with respect to erythrocytes of other species of animals was lower for free lipides and their acetone-soluble fraction than for combined lipides and their acetone-soluble fraction.

The hemolytic activity of lipides of the plague microbe and their acetone-soluble fractions, as in the case of lyophilized cultures and water-insoluble residues of the plague microbe, is higher in an acid environment than in an alkali one (Table 4).

Table 4

Influence of the pH of the Medium on the Hemolytic Activity of Lipides of the Plague Microbe (washed erythrocytes of the guinea pig; incubation at 37 degrees)

Medium	pH	Hemolytic activity in arbitrary units (based on 0.001 mg of preparation)			
		Free lipides		Combined lipides	
		Initial	Acetone-soluble fraction	Initial	Acetone-soluble fraction
Physical solution	6.98-7.04	8,955	12,583	9,959	14,284
Physical solution + 0.16M phosphate buffer	5.71	6,961	8,643	7,191	9,123
	7.83	10,319	15,112	11,412	18,191
	7.82	13,118	31,263	17,010	36,388

The lipides of the plague microbe and their acetone-soluble fractions, like washed aerated cultures of the plague microbe before and after lyophilization and also like water-insoluble residues of these cultures, lost their hemolytic properties in the presence of protein. [See Note] Thus in the presence of native sera of the horse, guinea pig, and horse gamma globulin hemolysis at 37 degrees did not occur during the course of 3 hours. Egg albumin was not as strong an inhibitor: under the same conditions hemolysis caused by free and combined lipides of the plague microbe reached 24-25% and hemolysis caused by the acetone-soluble fractions reached 11-14%.

[Note]: The final concentration of all protein preparations in the test as determined with a refractometer was equal to 0.75%.

Calcium and magnesium ions and cholesterine also suppressed the hemolytic activity of lipides of the plague microbe and their acetone-soluble fractions (Table 5).

Table 5

The Influence of Cholesterine and Calcium and Magnesium Ions on the Hemolytic Activity of Lipides of the Plague Microbe (washed erythrocytes of the guinea pig; incubation at 37 degrees)

Medium	Hemolytic activity in arbitrary units (based on 0.01 mg of preparation)			
	Free lipides		Combined lipides	
	Initial	Acetone-soluble fraction	Initial	Acetone-soluble fraction
Physical solution (control)	9,270	12,514	9,959	14,805
Physical solution + $3.25 \cdot 10^{-3}$ m Ca	1,854	2,503	1,904	2,867
Physical solution + $.235 \cdot 10^{-3}$ m Mg	2,360	3,305	2,503	3,949
Physical solution + 0.29% cholesterine	3,288	4,533	3,526	5,213

Remark: The portions of cholesterine which were used were first emulsified in a prepared 0.1% emulsion of the lipides being tested in the physical solution.

As indicated by the data of Table 5, the calcium ions have a more expressed inhibiting effect on the hemolytic activity of the tested preparation of lipides of the plague microbe than do magnesium ions.

Conclusions

Solubility in acetone and also in other organic solvents, weakening of the hemolytic activity by an excess of hydrogen ions and its intensification in an alkali medium, the inhibiting action of protein, cholesterine, and calcium and magnesium ions, thermal stability, etc. -- all these properties compare plague hemolysin with the higher fatty acids which are able to cause hemolysis.

Based on published data indicating that higher fatty acids such as palmitic, stearic and especially oleic acids are able to cause hemolysis in vivo (Greisman, 1958, 1959, etc.), it is possible to assume that plague hemolysin which is also a higher fatty acid is able in principle to display hemolytic properties when introduced in animals.

However, in connection with the fact that the lipide exchange of the plague microbe has hardly been studied at all, further identification of plague hemolysin as a higher fatty acid presents definite difficulties

and could be the subject of special investigations with respect to the lipide exchange of the plague microbe. Here we can only note that plague hemolysin is apparently a higher fatty acid of an unlimited series of the type of oleic acid which, as is known, (Greisman, 1959, etc.), is hemolytically more active than the limited fatty acids such as palmitic or stearic acids.

Inasmuch as we were able to come close to solving the chemical nature of hemolysin of the plague microbe the way is now open for studying its role in the pathogenesis of plague and for determining the mechanism of hemolysis in the case of this infection.

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