STUDIES OF THE BACTERICIDAL ACTION OF PHAGOCYTIN

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The preceding communication (1) described the extraction from rabbit polymorphonuclear leucocytes of a bactericidal substance which has been called phagocytin. This material manifests *in vitro* striking lethal action on various bacteria, especially on Gram-negative enteric bacilli. Phagocytin appears to be a protein with general properties characteristic of a globulin.

The present report deals with the effect of certain environmental conditions on the interaction between phagocytin and susceptible microorganisms.

Materials and Methods

The techniques employed for preparation of leucocytes from peritoneal exudates, for extraction of phagocytin from these cells, and for assay of bactericidal activity were identical with those described in detail in the preceding report (1).

RESULTS

The Relationship between the pH of the Medium and the Bactericidal Activity of Phagocytin.—

Since indicators show an acid reaction in the cytoplasm surrounding a phagocyted particle (2), it was of interest to determine whether the pH of the medium influenced the bactericidal action of phagocytin.

TABLE I

Relationship between the pH of the Medium and the Bactericidal Action of Phagocytin

Dilution of phagocyte extract in ICS medium	Percentage of *Escherichia coli* B killed in 1 hr. at 38°C. in ICS medium at pH						
	4.0	4.5	5.0	5.6	6.0	6.5	7.0
1:10	100	100	100	100	100	100	100
1:20	100	100	100	100	100	98	92
1:40	100	100	100	100	100	94	0
1:100	100	100	100	100	100	0	0
1:200	100	100	100	90	60	0	0
1:400	100	100	100	70	0	0	0
1:800	100	95	75	0	0	0	0
1:1600	100	70	0	0	0	0	0
1:3200	95	0	0	0	0	0	0
1:6400	0	0	0	0	0	0	0

Intracellular salt solution (ICS, see preceding communication for composition) was adjusted to the pH values indicated in Table I by addition of 1 N NaOH or of 1 N HCl. These solutions were sterilized in the autoclave and employed as suspending media for bactericidal tests.

As shown in Table I, a striking relationship existed between the pH of the medium and the bactericidal activity of phagocytin on *Escherichia coli* B. The more acid the environment, the more marked was the lethal effect. Observations could not be made in media more acid than pH 4.0, since under these conditions death of the microorganisms ensued even without the addition of leucocyte extract. A similar but less marked pH effect was noted when *Shigella sonnei* or *Klebsiella pneumoniae* C were studied in the same fashion.

The relationship between acidity and bactericidal activity of phagocytin in a medium other than ICS solution is shown in Table II.

TABLE II

Bactericidal Action of Extracts of Rabbit Polymorphonuclear Leucocytes in Albumin, Glucose, Acetate, Phosphate (AGAP) Medium at Various Reactions

Dilution of saline phagocyte extract in AGAP	Percentage of *Escherichia coli* B killed in 1 hr. at 38°C.		
	pH 5.0	pH 6.0	pH 7.0
1:40	100	100	100
1:80	100	100	91
1:160	100	97	0
1:320	100	58	0
1:640	80	0	0
1:1280	60	0	0
Dilution of saline phagocyte extract in AGAP	**Percentage of *Shigella sonnei* killed in 1 hr. at 38°C.**		
	pH 5.0	pH 6.0	pH 7.0
1:20	100	97	100
1:40	98	91	91
1:80	100	76	0
1:160	98	0	0
1:320	91	0	0
1:640	0	0	0
Dilution of saline phagocyte extract in AGAP	**Percentage of *Klebsiella pneumoniae* killed in 1 hr. at 38°C.**		
	pH 5.0	pH 6.0	pH 7.0
1:8	99	99	99
1:16	98	98	89
1:32	97	95	78
1:64	95	0	0
1:128	78	0	0
1:256	0	0	0

The medium containing albumin, glucose, acetate, and phosphate (AGAP) was prepared in the following fashion. The buffer solution consisted of equal parts of 0.05 M acetic acid–sodium acetate and of 0.05 M mixed phosphate at the three pH values indicated in Table II. The mixed buffers at pH 7.0 were sterilized in the autoclave, while those at pH 6.0 and 5.0 were used without sterilization so as to avoid possible loss of acetic acid on heating. These latter solutions were found to be free of contaminating bacteria detectable in the test system. To the buffer solutions were added sterile solutions of glucose and of bovine plasma albumin so as to produce a final concentration of 0.2 per cent glucose and 0.05 per cent albumin.

The results presented in Table II demonstrate that a bactericidal effect was exerted by the leucocyte extract in a medium composed of albumin, glucose, acetate, and phosphate (AGAP) on all three of the enteric bacilli studied. Moreover, as was the case in ICS medium, the bactericidal activity was enhanced at more acid reactions.

The Influence of the Composition of the Medium on the Bactericidal Activity of Phagocytin.—

As indicated by the findings presented immediately above, leucocyte extracts killed enteric bacilli in media other than ICS solution. Table III shows the effect on bactericidal activity of several additional alterations in test

TABLE III

Bactericidal Activity of Phagocytin in Various Media

Dilution of saline phagocyte extract in media at right	Percentage of *Escherichia coli* B killed in 1 hr. at 38°C. in					
	ICS	ICS containing 1 mg./ml. of EDTA- Na2*	ICS containing 5 mg./ml. glucose	ICS containing 1 mg./ml. enzymatic casein hydrolysate	0.1 M sodium citrate-HCl pH 5.6	0.1 M mixed phosphate buffer pH 5.6
1:100	100	100	100	100	100	100
1:200	98	98	100	94	100	98
1:400	56	94	92	73	100	0
1:800	0	50	0	0	98	0
1:1600	0	0	0	0	0	0

* Disodium salt of ethylenediaminetetraacetic acid.

media. It is seen that the cell extract killed *Escherichia coli* B in ICS solution, in a sodium citrate–HCl buffer at pH 5.6, or in phosphate buffer pH 5.6. The addition to ICS solution of the chelating agent ethylenediaminetetraacetic acid (EDTA), of glucose, or of enzymatic hydrolysate of casein brought about no significant change in the bactericidal action of phagocytin. The activity in citrate buffer, and in the presence of a chelating agent suggested that divalent cations were not required. However, since the tests were performed at an acid reaction in which binding of cations by citrate and EDTA was minimal, no certain conclusions could be drawn. Studies of the effect of a chelating agent on the killing of enteric bacilli by phagocytin in a neutral medium are presented in Table IV.

The following medium was used in these tests. A 0.05 M solution of monobasic potassium phosphate was adjusted to pH 7.0 with 1 N NaOH. To this solution was added, when indicated, the disodium salt of ethylenediaminetetraacetic acid at a final concentration of 0.2 per cent. After autoclaving the buffer mixtures, glucose was added in a final concentration of 0.2 per cent.

TABLE IV

Evidence That Divalent Cations Are Not Necessary for the Bactericidal Action of Phagocytin

Dilution of phagocyte extract in media at right	Percentage of bacteria killed in 1 hr. at 38°C.			
	Escherichia coli B		*Klebsiella pneumoniae* C	
	Phosphate-glucose medium pH 7.0	Phosphate-glucose-EDTA-Na2* medium pH 7.0	Phosphate-glucose medium pH 7.0	Phosphate-glucose-EDTA-Na2* medium pH 7.0
1:2	100	100	100	100
1:4	100	100	88	100
1:8	100	100	0	55
1:16	100	100	0	0
1:32	70	100	0	0
1:64	0	82	0	0

* Disodium salt of ethylenediaminetetraacetic acid.

As is demonstrated in the table, the bactericidal activity of phagocytin on both *Escherichia coli* B and on *Kelbsiella pneumoniae* C in a medium at neutrality was, if anything, slightly enhanced by the addition of a chelating agent. No killing of the bacteria resulted from exposure to the buffer solution containing EDTA in the absence of phagocyte extract. The results thus demonstrated that divalent cations were not required for the action of phagocytin, and stand in contrast to the strict requirement for magnesium ions in the properdin system (3, 4).

The slight enhancement of phagocytin activity in the presence of a chelating agent suggested that an excess of divalent cations might be antagonistic. An experiment designed to test this point is presented in Table V. The leucocyte extract used in this investigation was dialyzed against several large volumes of citrate-HCl buffer in order to remove, in so far as possible, divalent cations derived from the cells. It is seen that the lethal effect of phagocytin on *Escherichia coli* B and on *Shigella sonnei* was reduced slightly by a moderate concentration of magnesium sulfate (0.01 M, the concentration of magnesium ions contained in ICS solution, and approximately the concentration present inside phagocytic cells). A high concentration of magnesium sulfate (0.1 M) brought about striking reduction in, but not abolition of bactericidal activity. Since the medium employed for these tests contained citrate, the proportion of added salt existing as free magnesium ions was uncertain. It seems unlikely, however, that citrate would bind significant amounts of magnesium at this acid reaction.

TABLE V

Antagonistic Effect of Magnesium Ions on the Bactericidal Action of Phagocytin

Dilution of phagocyte extract, dialyzed against 0.1 M sodium citrate-HCl pH 5.6, in media at right	Percentage of bacteria killed in 1 hr. at 38°C.							
	Escherichia coli B in 0.1 M sodium citrate-HCl pH 5.6 containing				*Shigella sonnei* in 0.1 M sodium citrate-HCl pH 5.6 containing			
	No addition	0.01 M $MgSO_4$	0.1 M $MgSO_4$	0.4 M NaCl	No addition	0.01 M $MgSO_4$	0.1 M $MgSO_4$	0.4 M NaCl
1:10	100	100	100	100	100	100	78	96
1:20	100	100	100	100	100	100	72	100
1:40	100	100	100	100	98	94	74	100
1:80	100	100	82	100	90	78	0	88
1:160	100	100	62	100	50	0	0	86
1:320	100	100	0	100	0	0	0	0
1:640	100	100	0	100	0	0	0	0
1:1280	99	99	0	100	0	0	0	0
1:2560	98	87	0	96	0	0	0	0
1:5120	0	0	0	0	0	0	0	0

The lack of influence of a high concentration of sodium chloride (0.4 M) on phagocytin activity indicated that neutralization by magnesium salt was not merely due to the resulting high ionic strength of the medium. Other studies revealed that a high concentration of calcium ions manifested, in an acetate buffered medium, a neutralizing effect similar to that of magnesium ions. The examination of other metal cations for like effect was not possible, since high concentrations of them exerted a bactericidal action *per se*.

Table VI shows the influence of serum proteins on the bactericidal activity of phagocytin.

TABLE VI

Influence of Serum Proteins on the Bactericidal Activity of Phagocytin

Dilution of phagocyte extract in ICS medium	Percentage of *Shigella sonnei* killed in 1 hr. at 38°C. in ICS medium containing a final concentration of				
	10 per cent rabbit serum	1 per cent bovine albumin	0.1 per cent bovine albumin	1 per cent bovine globulin	No added protein
1:4	100	0	100	100	100
1:8	97	0	100	100	100
1:16	97	0	100	97	97
1:32	94	0	91	50	97
1:64	0	0	0	0	0

The phagocyte extract used in this experiment was derived from 2×10^9 polymorphonuclear leucocytes which were extracted with 25 ml. of 0.1 M sodium citrate adjusted to pH 5.6 by adding 1 N HCl. The soluble portion was then dialyzed against several volumes of the citrate-HCl buffer.

Normal rabbit serum was diluted in ICS solution and adjusted to pH 5.6. Bovine albumin and bovine gamma globulin (Armour and Co., Chicago) were dissolved at 1 per cent concentration in ICS solution and the pH adjusted to 5.6. These protein solutions were then sterilized by filtration through porcelain candles.

The presence of 10 per cent rabbit serum or of 1 per cent bovine gamma globulin in the medium did not significantly alter the bactericidal activity of the leucocyte extract. High concentrations of bovine albumin, however, blocked the lethal effect.

TABLE VII

Antagonistic Effect of Bovine Albumin on the Bactericidal Action of Phagocytin

Dilution of phagocyte extract in ICS containing bovine plasma fraction V as listed at right	Percentage of *Escherichia coli* B killed in 1 hr. at 38°C. in medium containing a final concentration of plasma albumin (pH 5.6) of			
	1.25 per cent	0.625 per cent	0.312 per cent	0.156 per cent
1:2	0	68	100	100
1:20	0	0	100	100
1:200	0	0	80	100

The experiment presented in Table VII was performed to clarify further the relationship between plasma albumin and antagonism of phagocytin. It is seen that the amount of phagocytin required for a given bactericidal effect was not directly proportional to the albumin concentration. At levels of albumin higher than approximately 0.5 per cent, little killing took place regardless of the quantity of leucocyte extract added, while activity was apparently fully restored when the albumin concentration was reduced to 0.3 per cent or lower.

The Relationship between the Concentration of Bacteria and the Bactericidal Action of Phagocytin.—

As is shown in Table VIII, a large increase (10,000-fold) in the numbers of *Escherichia coli* B resulted in only a small increase (2- to 4-fold) in the concentration of phagocytin required to kill more than 90 per cent of them. In those specimens containing a large inoculum, the end point tended to be less sharp, and small numbers of bacteria survived in the presence of even high concentrations of leucocyte extract. These small numbers of bacteria which survived did so by some means other than the development of inheritable resistance, for subcultures of them showed susceptibility to phagocytin identical to that of the original strain.

The Influence of Temperature and of Duration of Exposure on the Bactericidal Action of Phagocytin.—

The time-temperature relationships concerned with the killing of *Escherichia coli* B by phagocytin are presented in Table IX. It is seen that essentially no

TABLE VIII

Influence of the Concentration of Bacteria on the Bactericidal Activity of Phagocytin

Dilution of phagocyte extract in ICS medium	No. of surviving *Escherichia coli* B after 1 hr. at 38°C.		
	Final concentration of overnight culture inoculated		
	10^{-4}	10^{-5}	10^{-7}
1:16	1	0	0
1:32	10	0	0
1:64	26	0	0
1:128	70	0	0
1:256	3000	6	4
1:512	100000	1000	10
None	100000	1000	10
Dilution of phagocyte extract in ICS medium	**No. of surviving *Shigella sonnei* after 1 hr. at 38°C.**		
	Final concentration of overnight culture inoculated		
	10^{-5}	10^{-6}	10^{-7}
1:4	4	0	0
1:8	29	2	0
1:16	51	12	0
1:32	150	23	0
1:64	250	37	6
1:128	1000	100	10
None	1000	100	10

TABLE IX

Influence of Duration of Exposure and of Temperature on the Bactericidal Action of Rabbit Polymorphonuclear Leucocyte Extracts

Dilution of phagocyte extract in ICS	Percentage of *Escherichia coli* B killed											
	0°C.				27°C.				38°C.			
	5 min.	30 min.	1 hr.	4 hrs.	5 min.	30 min.	1 hr.	4 hrs.	5 min.	30 min.	1 hr.	4 hrs.
1:20	0	0	70	72	100	100	100	100	100	100	100	100
1:40	0	0	52	62	98	100	100	100	100	100	100	100
1:80	0	0	0	0	95	99	100	100	100	100	100	100
1:160	0	0	0	0	0	99	99	98	97	100	100	100
1:320	0	0	0	0	0	0	0	0	0	79	80	75
1:640	0	0	0	0	0	0	0	0	0	0	0	0

bactericidal action took place at 0°C. At 27°C. and at 38°C., a lethal effect was exerted on the bacilli rapidly, being well advanced in 5 minutes and complete within 30 minutes.

DISCUSSION

The enhanced bactericidal activity of phagocytin at acid reactions is of special interest in view of previous studies showing the pH about phagocyted particles to be quite low, probably in the vicinity of pH 4.5 (2). Thus intracellular conditions, at least in so far as the reaction is concerned, would appear to be highly suitable for killing of engulfed microorganisms by phagocytin.

Little alteration of the bactericidal activity of phagocytin resulted from several changes in the medium, such as variations in the buffer or in the ionic strength, the addition of glucose or of casein hydrolysate, and inclusion of metal binding agents. Of several proteins added to the medium, only plasma albumin influenced the action of leucocyte extracts on enteric bacilli. Final concentrations of bovine albumin in excess of 0.5 per cent seemed to block completely the bactericidal effect. Since phagocytin is known to be reasonably stable in solutions containing even 5 per cent albumin (1), it seems unlikely that this protein exerted its antagonism by attacking phagocytin; more likely albumin may somehow prevent combination between the bacteria and phagocytin. This neutralization by albumin does not mitigate against possible action of phagocytin *in vivo*, since in preliminary studies paper strip electrophoresis of concentrated leucocyte extracts has revealed no large amounts of any protein resembling serum albumin.

The experiments employing chelating agents established that divalent cations were not required for the lethal action of phagocytin on bacteria, and in fact, led to the demonstration that high concentrations of magnesium or calcium ions neutralized this action. It should be pointed out that the concentration of these cations necessary to antagonize phagocytin significantly was far in excess of that present within phagocytic cells.

Although further studies must be done to establish the mechanism by which phagocytin affects the susceptible microorganisms, the quantitative relationships between numbers of bacteria and the concentration of phagocytin required to kill them, and the time-temperature characteristics of the lethal action are both in keeping with an enzymatic reaction. The fact that enteric bacilli are not attacked by phagocytin when exposed at 0°C. might also be interpreted as indicating that only microorganisms engaged in active metabolism manifest susceptibility to this agent.

SUMMARY

The bactericidal activity of phagocytin on Gram-negative enteric bacilli is influenced by the reaction of the medium; the more acid the environment, the more marked is the activity.

Phagocytin exerts approximately the same action whether citrate, acetate, or phosphate salts are used as buffer, and the addition of glucose, casein hydrolysate, or cation binding agents does not produce notable change. Although

proteins in general have but little effect, the inclusion in the medium of a high concentration of bovine plasma albumin neutralizes the lethal action of phagocytin on enteric bacilli. Very high concentrations of magnesium or calcium ions antagonize but do not completely block the bactericidal effect.

The bactericidal activity of phagocytin is essentially independent of the numbers of bacteria exposed; a 100-fold increase in the numbers of enteric bacilli results in approximately a 2-fold increase in the concentration of phagocytin required to kill 90 per cent of them.

When susceptible microorganisms are exposed to phagocytin at 0°C., practically no killing takes place. At higher temperatures the bactericidal action is rapid, being well advanced in 5 minutes and complete within 30 minutes.

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