BACTERICIDAL ACTIVITY OF RAT LEUCOCYTIC EXTRACTS

I. Antibacterial Spectrum and the Subcellular Localization of the Bactericidal Activity*

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Ever since the phenomenon of phagocytosis has been observed, investigators have been trying to determine the substance or substances within the white blood cells which are responsible for the observed bactericidal activity. Through the course of years, the "digestive" phase of phagocytosis was attributed to enzymatic action. Yet, none of the proteolytic enzymes, present in white blood cells, exhibit bactericidal activities. A mucopolysaccharidase, lysozyme, present in polymorphonuclear neutrophils (leucocytes) does possess bactericidal activity against several bacterial species. However, according to our present state of knowledge, the antibacterial spectrum of this enzyme is limited and at best lysozyme would only contribute a minor part to the overall process of phagocytosis. Therefore it is feasible to consider the "digestive" phase of phagocytosis as a result of a non-enzymatic bacteridical substance which when coupled with proteolytic enzymes leads to the observed bacterial breakdown.

In the present investigation, the bactericidal activity of extracts prepared from rat leucocytes was studied. It had been previously shown (1) that such an extract prepared by means of sonic vibration did exhibit some killing against *Micrococcus pyogenes* var. *aureus* (*M. aureus*) when tested *in vitro*. The active substance or substances was found to be thermolabile, non-dialyzable, fractionable with calcium phosphate gels or $(NH_4)_2SO_4$, and to have an optimum activity at pH 7.5. It has now been observed that leucocytic extracts prepared by means of ultrasonic vibration possess an even more pronounced bactericidal effect against *M. aureus*. The bactericidal substance in this case differs from that obtained with sonic vibration in some of its physical-chemical properties, as will be described below. All experiments reported here were conducted with extracts of rat leucocytes prepared by ultrasonic vibration.

* The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official, or reflecting the views of the Navy Department.

Materials and Methods

Experimental Animals.—250 to 350 gm. male and female Sprague-Dawley rats were used throughout this study.

Preparations of Polymorphonuclear Leucocytes.—Leucocytes were obtained from the peritoneal cavity of animals injected 12 to 18 hours previously with an aleuronat-starch-tryptose broth mixture (1). After washing the leucocytes three times with M/15 phosphate buffer solution, pH 7.0, the leucocytes were suspended in 10 ml. of the cold buffered solution and their concentration was determined by means of a hemocytometer. The leucocytes were broken up by means of a 1000 kc. crystalab ultrasonic vibrator; 1000 V, 130 ma. and the extract was filtered through a Seitz S-1 filter. In some experiments the extract was dialyzed against distilled water for 24 hours before being filtered.

Mitochondrial Extracts.—The subcellular components of leucocytes were separated by the differential centrifugation method of Schneider *et al.* (2) as modified by Cole *et al.* (3), in that the suspending medium consisted of:—

A. Salt Solution:

KH_2PO_4	0.0094M
K_2HPO_4	0.0125M
$NaHCO_3$	0.0015M

B. To 100 ml. of the salt solution were added:—

Sucrose	8.2837 gm.
Glucose	100 mg.
ATP	20 mg.

The suspended leucocytes were disrupted in the cold by means of a Potter glass homogenizer. The various subcellular fractions were separated by differential centrifugation at the following speeds: Fraction I (nuclei) 600 *G*/10 minutes, Fraction II (mitochondria) 8500 *G*/10 minutes, Fraction III (microsomes) 20,000 *G*/90 minutes, and Fraction IV consisting of the remaining supernatant. All fractions including Fraction IV were exposed to ultrasonic vibration before being filtered through a Seitz filter.

Bacterial Cultures.—All bacterial species were grown as a broth culture for 24 hours at 37°C. The bacterial cells were collected at 3000 R.P.M. for 20 minutes and the packed cells resuspended in sterile saline to a given volume so that 0.1 ml. of the suspension when mixed with 2.5 ml. of saline would give 100 to 200 colonies per 0.1 ml. when plated.

Bactericidal Assay.—All leucocytic or mitochondrial extracts were prepared from approximately 4×10^8 polymorphonuclear neutrophils. The extracts were diluted initially 15-fold with phosphate buffer solution, pH 7.5, and constituted the standard extract solution. Further 2-fold dilutions were made of the standard solution (up to 1:480) and 2 ml. of each dilution were dispensed into sterile serological test tubes. To each test tube were added 0.5 ml. of a 10^{-5} dilution of *M. aureus* suspension and 0.2 ml. of a 0.2 M $CaCl_2$ solution. The mixtures were incubated at 37°C. and at 0, 30, and 60 minutes, 0.1 ml. aliquots of each tube were spread on a nutrient agar plate. The plates were incubated overnight at 37° C. The number of colonies were counted and expressed as the number of bacteria per 0.1 ml.

Appropriate dilutions were made of the other bacterial species tested so as to give approximately 100 to 200 colonies when 0.1 ml. of the assay mixture was plated directly on an agar plate.

Nitrogen Determinations.—The Markham modification of the micro-Kjeldahl procedure (4) was used to determine the nitrogen content of both the leucocytic and mitochondrial extracts. The amount of nitrogen in these extracts was expressed as milligrams of nitrogen per milligram of leucocytes (dry weight).

RESULTS

Leucocytes obtained from 10 rats previously injected with the aleuronat mixture, were pooled and diluted with phosphate buffer solution to give a final concentration of 4×10^8 leucocytes per 15 ml. The extracts prepared from these cells were tested for bactericidal activity against *M. aureus*. The results of a typical experiment are seen in Table I. It can be noted that there was

TABLE I

Bactericidal Activity of Leucocytic Extract (LE) from Rats Against M. aureus

Dilution of LE	Colonies per 0.1 ml. after incubation			Per cent killing after incubation for	
	0 min.	30 min.	60 min.	30 min.	60 min.
1:15	99	13	0	87	100
1:30	124	43	0	65	100
1:60	112	75	3	33	97
1:120	114	80	11	30	90
1:240	104	94	17	9.6	84
1:480	98	94	39	3.8	60
—	113	112	112	0	0

TABLE II

Influence of Bacterial Concentrations on the Bactericidal Activity of Leucocytic Extracts from Rat

No. of bacteria/0.1 ml. after incubation for:		Per cent killing after incubation for:
0 min.	60 min.	60 min.
2,980,000	7,000	96
212,000	0	100
14,600	0	100
2,000	0	100
194	0	100
187*	184	0

* Bacterial control.

progressive killing of the organisms with increased incubation time. At the end of 60 minutes of incubation, a 1:240-fold dilution of the extract killed 84 per cent of the bacteria.

The influence of bacterial concentration on the bactericidal activity of the undiluted leucocytic extract was determined by adding varying concentrations of *M. aureus* to a constant amount of leucocytic extract. Tenfold dilutions of *M. aureus* were added to a constant amount of the leucocytic extract prepared from approximately 4×10^8 leucocytes. The results are tabulated in Table II. From this data the total number of bacteria killed was calculated to be 2.97×10^7 per ml.

Intercellular Localization of Bactericidal Activity.—In order to determine whether the bactericidal activity of rat leucocytes is confined to any particular morphological subfraction of the cell, the leucocytes were homogenized and

TABLE III

The Bactericidal Activity of Extracts Prepared from Subcellular Components of Rat Leucocytes

Subcellular fraction	Dilution of extract	Colonies per 0.1 ml after incubation for:		Per cent killing after incubation for:
		0 min.	30 min.	30 min.
Nuclei	1:15	207	5	98
"	1:30	192	50	74
"	1:60	179	115	36
"	1:120	189	158	16
"	1:240	159	150	5.7
"	1:480	156	149	4.5
Mitochondria	1:15	162	0	100
"	1:30	182	0	100
"	1:60	180	0	100
"	1:120	192	29	85
"	1:240	189	55	71
"	1:480	163	100	38
Microsomes	1:15	226	0	100
"	1:30	195	5	97
"	1:60	202	130	36
"	1:120	189	185	2.1
"	1:240	198	192	3.0
"	1:480	230	226	1.7
Soluble fraction	1:15	240	236	1.6
" "	1:30	231	228	1.3
" "	1:60	215	219	0
" "	1:120	236	239	0
" "	1:240	242	238	1.6
" "	1:480	230	235	0

the nuclear, mitochondrial, and microsomal fractions were separated by means of differential centrifugation as described above. These fractions, including the soluble fraction, were individually assayed for their bactericidal activity. The results as seen in Table III clearly indicate that the bactericidal activity of leucocytes was confined to the mitochondrial fraction. After 30 minutes of incubation at 37°C., a 1:120-fold dilution of the mitochondrial extract gave 85 per cent killing. The highest dilutions of the nuclear and microsomal fractions which gave 80 to 100 per cent killing were respectively 1:15- and 1:30-fold dilution. No bactericidal activity was observed with the soluble fraction.

The slight bactericidal activity present in both the nuclear and microsomal fractions could be accounted for by mitochondrial contamination.

A comparison between the relative bactericidal activity of the leucocytic extracts and the mitochondrial extracts was made. In this experiment, ap-

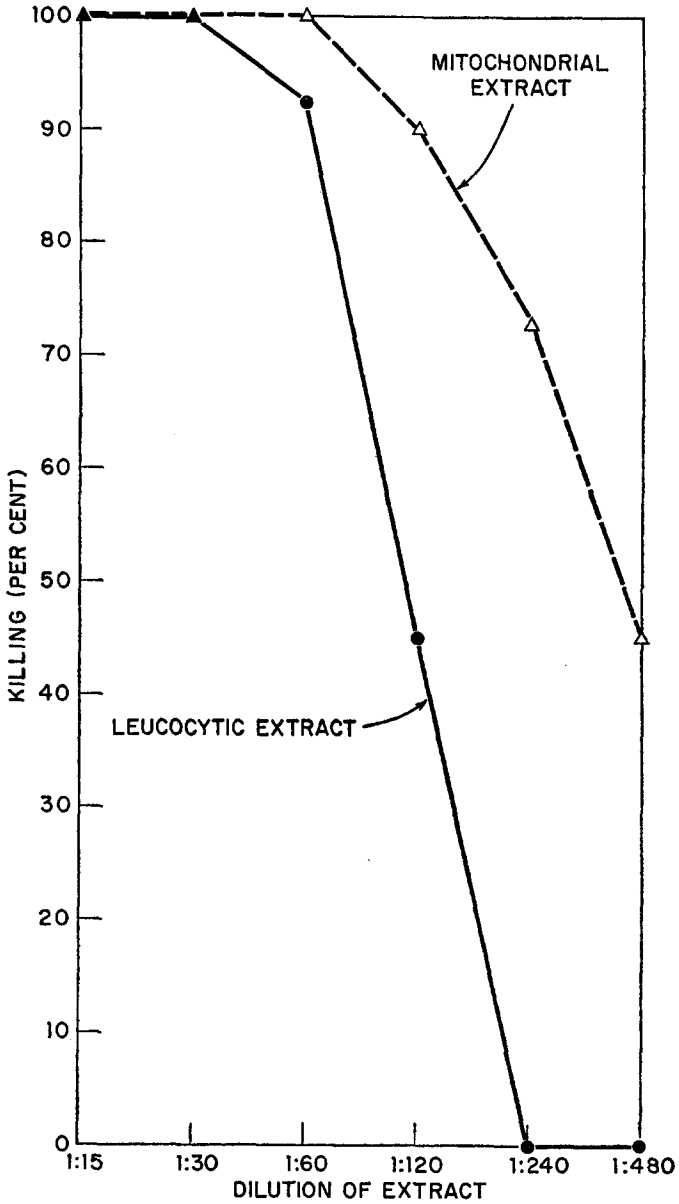

FIG. 1. The bactericidal activity of leucocytic mitochondrial extract against *M. aureus*.

proximately 8×10^8 leucocytes were collected from the peritoneal cavities of 30 rats. A whole leucocytic extract was prepared from an aliquot containing 4×10^8 leucocytes, and the remaining cells were used to obtain a mitochondrial extract. Both extracts were assayed for their bactericidal activity and

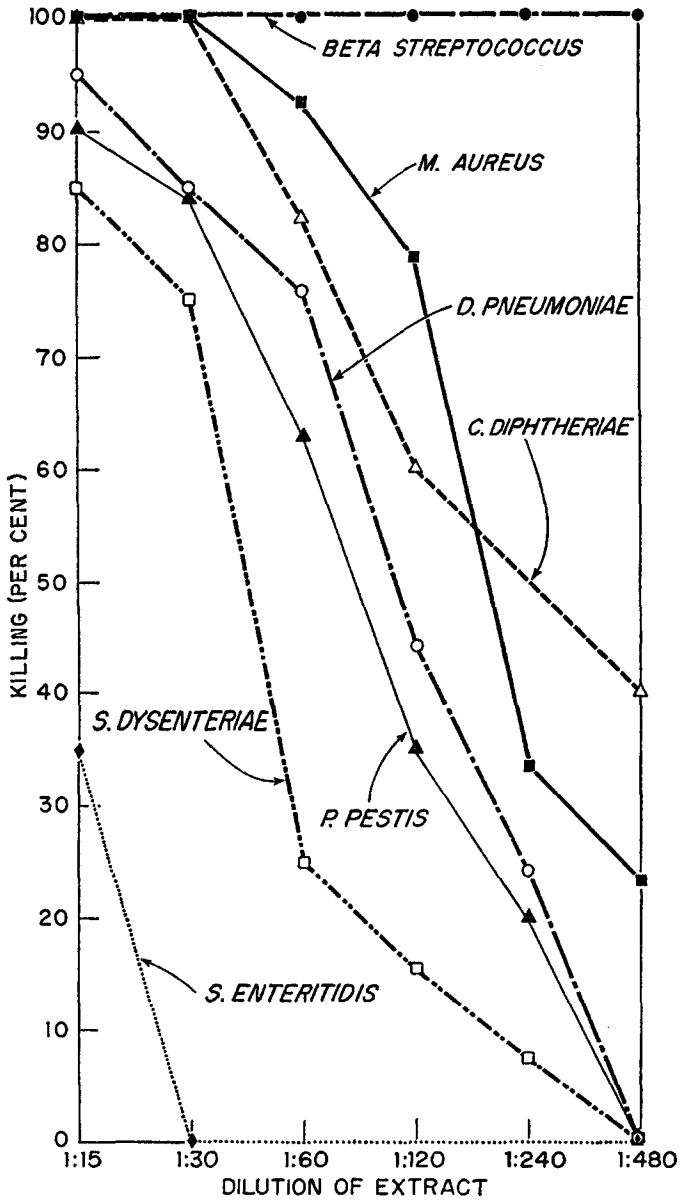

FIG. 2. Bactericidal activity of mitochondrial extracts against several species of bacteria.

the results are recorded in Fig. 1. The mitochondrial extract exhibited a slightly greater activity than that noted with the leucocytic extract. The highest dilution that gave 80 per cent killing was 1:120 for the mitochondrial extract and 1:60 for the leucocytic extract. However, the bactericidal activity of the mitochondrial extract in terms of N content, was significantly greater than that of the whole leucocytic extract. The results of the N determinations were as follows: dry weight of approximately 4×10^8 leucocytes was 18.9 mg.; the nitrogen content for the leucocytic extract was 3.1 μg. N per mg. of leucocytes, the nitrogen content for the mitochondrial extract was 0.65 μ g. N per mg. of leucocytes. Thus the *mitochondrial* extract activity represents a 5-fold purification step over the leucocytic extract. The amount of nitrogen present in the assay mixture containing a 1:15 dilution of the mitochondrial extract was 18.6 μg. of nitrogen per 2.5 ml.

Anti-Bacterial Spectrum.—

The antibacterial spectrum of the mitochondrial extract was determined by adding varying dilutions of the extract to a constant number of bacteria of the different species tested. The various organisms tested and the agar media used in the assays were as follows:

Streptococci (beta)	Blood agar
Diplococcus pneumoniae	Blood agar
Pasteurella pestis (strain A1122)	Proteose peptone No. III
Corynebacterium diphtheriae	Proteose peptone No. III
Shigella dysenteriae	Nutrient agar
Salmonella enteritidis	Nutrient agar
Micrococcus pyogenes var. *aureus*	Nutrient agar

It can be noted (Fig. 2) that both the Gram-positive and Gram-negative bacteria were killed by the extract. However, the Gram-positive organisms were more susceptible to the bactericidal activity of the mitochondrial extract than were the Gram-negative bacteria. In the course of these experiments other species of bacteria were found to be susceptible to the killing action of the mitochondrial extract; *i.e.*, an *Enterococci* strain, *Sarcina lutea* and an *Alcaligenes* strain.

DISCUSSION

By use of an *in vitro* technique it has been shown that rat leucocytic extracts exhibit a bactericidal activity against many bacterial species. The responsible bactericidal substance was found primarily localized in the mitochondria of the *leucocytes*. The activity of the mitochondrial extracts was very potent when measured in terms of nitrogen content. An extract containing as little as 0.31 μg. N was capable of killing approximately 2×10^3 cells of *M. aureus* and even a greater concentration of *β-Streptococci.*

Microscopically, the bacteria were not lysed by the mitochondrial extracts. Yet they were killed as evidenced by lack of growth after extended incubation in enrichment media. However, as yet no evidence is available as to the

mechanism of its action. One can postulate from these experiments that the "digestive" phase of phagocytosis in rat leucocytes involves at least two steps; a non-enzymatic killing of the engulfed organisms followed by "digestion" as the result of proteolytic attack on the dead bacteria.

Hirsch (5, 6) recently has reported the isolation of a bactericidal substance, phagocytin, present in rabbit but not in rat leucocytes. This substance was active against Gram-negative bacteria and showed little or no effect against Gram-positive organisms. Whether Phagocytin is or is not similar to the one reported here has not been fully established. The one difference that can be mentioned here is that the bactericidal substance isolated from rat leucocytes was active against both Gram-positive and Gram-negative organisms with the former group being more susceptible.

If one establishes the degree of invasiveness in rats of the bacteria tested in terms of a subcutaneous injection giving rise to a local abscess, generalized lesions and/or bacteriemia, it appears that the *in vitro* susceptibility of the organism to the leucocyte extract is inversely proportional to their invasive ability. For example, a subcutaneous injection of *M. aureus* results in a local suppurative lesion and this organism is highly susceptible to the leucocytic extract, whereas *Salmonella enteritidis* is highly invasive in the rat and is more resistant to the bactericidal activity of leucocytic extracts.

This generalization is of interest when one considers that the polymorphonuclear neutrophiles constitute the first line of defense following a subcutaneous injection.

SUMMARY

An extract of polymorphonuclear leucocytes of the rat, prepared by means of ultrasonic vibration, was found to be bactericidal against *M. aureus*. The bactericidal activity was primarily confined to the mitochondrial fraction of the leucocytes.

The rat leucocyte mitochondrial extract was bactericidal against both Gram-positive (*M. aureus*, *β-Streptococci*, *Diplococcus pneumoniae*, *Corynebacterium diphtheriae*) and Gram-negative (*Shigella dysenteriae*, *Salmonella enteritidis*, *Pasteurella pestis*) bacteria. The Gram-positive organisms were more susceptible to the bactericidal activity of the mitochondrial extracts.

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