PHAGOCYTIN: A BACTERICIDAL SUBSTANCE FROM POLYMORPHONUCLEAR LEUCOCYTES

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Ever since the classical reports of Metchnikoff over 50 years ago (1, 2), the importance of phagocytic cells in protecting animals from infectious diseases has been generally accepted. Practically no information is available, however, concerning the intraphagocytic biochemical conditions which bring about death of the engulfed microorganisms. Most textbooks simply state that parasites are "digested" in the phagocytic cytoplasm. This statement has little real meaning, for it has been shown that *living* bacteria are not affected by exposure to many of the common proteolytic enzymes (3). The only bactericidal material recognized to be present in phagocytes is lysozyme, an aminopolysaccharidase found in polymorphonuclear leucocytes (4–6). This enzyme can hardly be responsible for all of the antibacterial activity of these cells, since they are known to destroy many bacteria which are insusceptible to lysozyme.

The present communication describes studies leading to the characterization of another biochemical mechanism which may kill certain bacteria in the cytoplasm of polymorphonuclear leucocytes.

RESULTS

Preliminary Investigation of the Bactericidal Activity of Intact and of Disrupted Exudate Leucocytes.—

The first studies done were designed to determine the presence or absence of preformed bactericidal substances in acute phase exudate cells obtained from the rabbit.

Young adult New Zealand Red rabbits weighing 2500 to 3500 gm. were used. Exudates were induced by the intraperitoneal injection of either of two solutions: (*a*) 50 ml. of an equal mixture of 40 per cent gum acacia and 20 per cent bacto beef extract in 0.9 per cent NaCl, or (*b*) 50 ml. of a neutral solution of 10 per cent sodium caseinate in 0.9 per cent NaCl. Approximately 18 hours later the animals were sacrificed by exsanguination, and the peritoneal exudates were collected in heparinized (1:10,000) Hanks solution. In other experiments 0.1 M mixed phosphate buffer pH 7.4, or 0.1 M sodium citrate solutions were used with similar results. The exudates were spun at 1200 R.P.M. for 10 minutes at room temperature (International centrifuge, size 2). The cell buttons were then washed two times by suspending in buffered salt solution and centrifuging as described above. At the time of the final collection, the cell suspension was distributed into several 15 x 120 mm. screw cap tubes, thus giving mul-

tiple cell buttons of small volume. Sterile glassware and solutions were used in all operations.

In a representative experiment of this group, the results of which are presented in Table I, 8 washed cell buttons were obtained. One-half of these was stored at 4°C. until time for use, while the other half was subjected three times to alternate freezing in dry ice-acetone (−75°C.) and thawing in a 38°C. water bath. Microscopic observation of the cells maintained at 4°C. showed them to be approximately 80 per cent pseudoeosinophilic polymorphonuclear leucocytes. A variable amount of debris was always present. Supravital staining with trypan blue revealed that well over 60 per cent of the leucocytes in the specimens kept at 4°C. were alive, while, as expected, all the cells in buttons subjected to freezing and thawing appeared to be dead.

The bacteria used in these experiments were cultured in beef heart-peptone broth pH 7.4. After overnight incubation at 38°C., the bacterial cells were collected by centrifugation, and resuspended and diluted in appropriate buffer solution (heparinized Hanks, phosphate, or sodium citrate). One drop of a 1:10,000 dilution of the bacterial suspension was carefully added with a capillary pipette directly to a white cell button. After mixing with a glass rod, the inoculated buttons were incubated with constant agitation at 38°C. for 90 minutes. Next they were each diluted with 10 ml. of sterile distilled water and held at room temperature for 15 minutes. Volumes of 0.1 ml. and 9.9 ml. of this suspension were then passed through membrane filters (Millipore Filter Corp., Watertown, Massachusetts), and the numbers of surviving bacteria were determined by counting colonies on the filters after overnight incubation at 38°C. on beef heart-peptone agar plates (7).

TABLE I

Bactericidal Activity of Intact and of Disrupted Rabbit Peritoneal Exudate Cells

After incubation at 38°C. for 90 min. with	No. of surviving bacteria			
	Klebsiella pneumoniae type C	*Escherichia coli* B	*Staphylococcus albus*	*Streptococcus* H69D
Leucocyte button (intact cells)	0	0	50	2500
Frozen-thawed leucocyte button (disrupted cells)	4	0	0	4700
Heparinized Hanks solution only (no cells)	1200	2500	4000	3400

As is demonstrated in Table I, incubation of *Klebsiella pneumoniae* type C, *Escherichia coli* B or *Micrococcus pyogenes* var. *albus* with packed rabbit exudate cells led to death of nearly all the bacteria. Leucocytes which had been disrupted by freezing and thawing appeared to exert as marked a bactericidal effect as did the intact white cells. Neither living nor dead exudate cells killed a group D streptococcus included in the experiment.

When disrupted cell buttons were diluted approximately 3-fold with 0.1 M phosphate buffer pH 7.4, the suspension retained bactericidal activity on *Klebsiella pneumoniae* C. Heating at 100°C. for 10 minutes destroyed this activity. The stability on dilution in buffer, and the instability on heating indicated that the bactericidal effect was not simply due to the well recognized acid nature of phagocytic cytoplasm (8). Also, it seemed unlikely that the

lethal action on *Klebsiella pneumoniae* and on *Escherichia coli* B was due to lysozyme, since these microorganisms were not killed when incubated under similar conditions with high concentrations of crystalline egg white lysozyme (Armour and Co., Chicago). The results thus suggested that acute phase rabbit exudate cells contained a bactericidal substance or substances other than lysozyme or acid.

Two considerations made it desirable to develop a more suitable technique for collection of polymorphonuclear leucocytes before proceeding with studies of the antibacterial substances contained therein. First, as mentioned above, approximately 20 per cent of the white cells in the 18 hour peritoneal exudates were not polymorphonuclear ones. Secondly, further experimentation on exudates induced with beef extract or sodium caseinate gave results which were not satisfactorily reproducible. The new technique described below eliminated both of these objectionable features, and offered the additional advantage that sacrifice of the animal was not required.

A stock of 15 young adult New Zealand Red rabbits was utilized for all experiments to be described below. Peritoneal exudates were not collected from any individual animal more frequently than once weekly.

The solutions used for collecting and processing the exudate cells had the following composition. Saline: 26 ml. saturated solution of NaCl in distilled water, distilled water to 1000 ml. Citrate-saline: 4 gm. reagent grade sodium citrate dihydrate, 26 ml. saturated solution of NaCl in distilled water, distilled water to 1000 ml. "Lysis solution": 1 gm. sodium citrate dihydrate, 7 ml. saturated solution of NaCl in distilled water, distilled water to 1000 ml. The pH of this "lysis solution" was adjusted to 5.0 by adding 1 N HCl. "Neutralizing solution": 7 gm. sodium citrate dihydrate, 41 ml. saturated solution of NaCl in distilled water, distilled water to 1000 ml. All solutions were sterilized in the autoclave.

The intraperitoneal injection of large volumes of saline alone gave suitable exudates in some animals. But, as noted by previous workers (cited in reference 9), the numbers of cells so obtained varied considerably. It was found that highly cellular peritoneal exudates were formed in reproducible fashion when small amounts of glycogen (C.P., Amend Drug and Chemical Co., Inc., New York) were added to the saline solution. The glycogen (not sterilized) was added in a concentration of 1 mg. per ml. to the saline solution just prior to injection into the animal.

After the abdominal area had been clipped and the skin wiped with 70 per cent ethanol, 250 ml. of warm (38°C.) saline solution containing glycogen was injected intraperitoneally. The exudate was collected 4 hours later, using a device composed of the following elements. A number 13 needle, 2½ inches long with multiple perforations approximately 2 mm. in diameter bored through its shaft near the bevel, was connected by 2 feet of small rubber tubing (3 to 4 mm. internal diameter) to a 3 way stop-cock. Another portal of this stop-cock was attached to a 50 ml. syringe, and from the third portal was led a short length of small bore rubber tubing. The entire apparatus was rinsed with acetone and then with sterile distilled water before use. With the syringe and the tubing leading to the needle filled with citrate saline solution, the needle was introduced through the skin just cephalad to the bladder area in the midline, was then threaded in the subcutaneous tissue for about 1 inch, and was finally plunged into the peritoneum. The tip of the needle thus lay at the level of the umbilicus. The animal was fixed to a board with its ventral side downward. After 200 ml. of citrate saline solution warmed to body temperature was injected into the peritoneum with the syringe, the stop-cock was turned to the closed position and the rabbit's abdomen was gently kneeded. The stop-cock

was next opened between the tubing to the needle and the other length of rubber tubing. With the entire apparatus placed lower than the animal, the peritoneal exudate flowed gently by gravity. The flow was at times slow and intermittent, and could often be improved when the operator raised the animal with his hands and pooled the remaining fluid about the tip of the needle. After no further fluid could be obtained, an additional 100 ml. of citrate saline solution was introduced and collected in similar fashion. Rabbits rarely manifested any signs of discomfort during the introduction of the needle or during the 20 minutes or so of fixation required. In a series of approximately 100 such collections, there were no fatalities and no significant complications.

A total of 550 ml. of fluid was thus introduced, 250 ml. during the original injection, and 300 ml. at the time of collection. The yield varied from 350 to 450 ml. of a white or pinkish opalescent suspension. In an occasional animal gross blood contaminated the exudate. Even though debris was rarely visible, the exudate was poured through three layers of gauze. The volume was then recorded and a white cell count done, using ordinary hematological methods. Between 5×10^8 and 2×10^9 cells were obtained from each rabbit. A "wet" differential in the counting chamber revealed almost entirely polymorphonuclear cells.

The exudate was next placed in 200 ml. bottles with sterile rubber caps and spun at 1400 R.P.M. (International centrifuge, size 2) for 10 minutes at room temperature. To remove red cells, the buttons from each animal were well drained and then suspended in 20 ml. of the hypotonic, slightly acid "lysis solution." After standing 10 minutes at room temperature, 20 ml. of the hypertonic "neutralizing solution" was added. Centrifugation was repeated, this time in 25 x 150 mm. screw cap tubes at 1200 R.P.M. for 10 minutes. The sedimented material was then resuspended in citrate saline solution and spun again, using the same technique. The cell button thus obtained from each rabbit was a pearly white, creamy material 0.5 to 1.0 ml. in volume.

Clumping of the white cells in the final preparation made it impossible to do accurate counts; however it appeared that no significant loss of leucocytes resulted from the manipulations. Examination for viability, using lack of staining by trypan blue as a criterion therefor, showed 60 to 80 per cent living cells. Wright's stains on portions of the cell buttons washed and suspended in saline containing 20 per rabbit serum revealed at least 95 per cent, and in many cases over 99 per cent pseudoeosinophilic polymorphonuclear leucocytes. No red cells and little or no debris were seen. For the present studies, in which viability of cells was not the objective, the above method proved satisfactory. It is of course possible that additional care, for example the use of a more physiological salt solution and maintenance at low temperature during processing, might lead to "pure" preparations of leucocytes in an even better state of health.

The white cell buttons were stored at −21°C. until extracted for bactericidal tests. Except as otherwise noted in the text, they were extracted in a salt solution imitating, as well as present knowledge permits, the cationic composition of rabbit polymorphonuclear leucocytic cytoplasm (9, 10). This solution, hereafter referred to as ICS (intracellular salts), was made as follows: 7.5 gm. KH_2PO_4; 11.4 ml. of a saturated solution of KCl in distilled water; 2.46 gm. $MgSO_4 \cdot 7H_2O$; 3.85 gm. sodium citrate dihydrate; distilled water to 1000 ml. It had a pH of 5.6 and was sterilized in the autoclave. The leucocyte button was suspended in various amounts of this salt solution, ordinarily 5 to 10 ml. of ICS per 10^9 polymorphonuclear cells, and the suspension was then subjected to three cycles of freezing in dry ice–acetone (at least 10 minutes) and thawing in a 38°C. water bath (until just liquified). The disrupted cell suspension was next spun for 15 minutes at 3000 R.P.M. (size 2 International centrifuge, 50 ml. carriers), resulting in a small white button and a viscous opalescent supernatant. Wright's stains on the sediment showed eosinophilic granules, intact nuclei with bits of adherent cytoplasm, and homogeneous masses of faintly basophilic material.

The bactericidal activity was found to reside in the supernatant solution.

Stock cultures of bacteria were maintained by weekly transfer in beef heart-peptone broth pH 7.4. The bactericidal tests were performed in the following fashion. Serial dilutions of the cell extract were made in ICS solution (or in other media as will be pointed out in certain experiments) so that each of the sterile 13 x 100 mm. cotton-plugged test tubes contained a final volume of 0.4 ml. To each of these tubes was added 0.1 ml. of a dilute suspension of the microorganism, usually approximately a 10^{-6} concentration of an overnight culture. Care was taken not to leave hanging on the walls of the tubes drops of bacterial inoculum which would not be exposed to the cell extract. After incubation at 38°C. for 1 hour (except as otherwise noted in the text), the contents of each tube was mixed with a pipette and 0.1 ml. was spread on beef heart-peptone agar in Felsen plates. (These agar plates were incubated at 38°C. for 24 hours prior to use to rid them of excess surface moisture.) The plates were then incubated in an upright position at 38°C. overnight and colonies were counted to determine the numbers of surviving bacteria. Preliminary experiments established that no death of enteric bacilli took place in the ICS medium alone. Dilutions of the bacterial cultures were selected to yield 50 to 100 colonies from the control tubes containing no cell extract. This number of colonies was small enough to permit ready counting, yet sufficiently large to give statistically significant results. Variations as large as 2-fold occurred in counts on duplicate specimens. Therefore in those of the following tables in which results are presented as percentage of bacteria killed, any value of 50 per cent or less is recorded as 0.

Both the nature of the cellular exudate and the bactericidal activity of the extracts were satisfactorily reproducible when the above technique was employed. There was but little variation among individual animals; in exudates collected from each of the 15 rabbits on 6 to 9 occasions, the polymorphonuclear leucocyte yield ranged between 5×10^8 and 2×10^9, and the bactericidal activity obtained on each occasion, calculated on the basis of the highest dilution of an extract of 10^9 leucocytes which would kill *Escherichia coli* B under standard conditions, varied no more than 3-fold.

The Influence of the Nature of the Substance Employed to Promote Exudate Formation on the Bactericidal Activity of Leucocyte Extracts.—

In the standard procedure described above, glycogen and saline were injected intraperitoneally into rabbits, the glycogen serving as a chemotactic agent to call forth circulating phagocytes. Extracts of cells so obtained manifested striking bactericidal activity. Since the polymorphonuclear leucocyte is known to possess a glycolytic apparatus, it was uncertain whether this bactericidal activity was due to a preformed substance, or due to certain products, for example lactic acid, which might result from catabolism of glycogen. It was therefore of interest to use other substances as irritant or chemotactic materials and determine the antimicrobial effect of phagocytic extracts thereby obtained.

Potato starch (Morningstar, Nichol, Inc., New York) and bovine hemoglobin enzyme substrate powder (Armour Laboratories, Chicago), each added in a final concentration of 1 mg. per ml. to 0.9 per cent NaCl solution, were compared to glycogen as exudate inducing agents. The general techniques employed were identical with those described above.

TABLE II

Bactericidal Activity of Extracts of Rabbit Polymorphonuclear Leucocytes from Peritoneal Exudates Induced by the Injection of Various Substances

Dilution of phagocyte extract in ICS* medium	No.‡ of surviving *Escherichia coli* B after 1 hr. at 38°C.		
	Starch exudate	Glycogen exudate	Hemoglobin exudate
1:3	0	0	0
1:30	0	0	0
1:300	0	0	0
1:3,000	70	50	9
1:30,000	150	150	150
None	150	150	150
	No.‡ of surviving *Shigella sonnei* after 1 hr. at 38°C.		
	Starch exudate	Glycogen exudate	Hemoglobin exudate
1:3	0	0	0
1:9	0	0	0
1:27	24	11	1
1:81	150	100	26
1:243	150	300	250
None	150	300	250

* ICS, intracellular salts.

‡ Per 0.1 ml. of bacteria-leucocyte extract mixture.

The numbers and types of white cells obtained were not detectably different when potato starch or hemoglobin was used as irritant in place of glycogen. And, as is shown in Table II, extracts of polymorphonuclear leucocytes from animals given these materials all manifested similar bactericidal activity on *Escherichia coli* B and on *Shigella sonnei.*

Technique for Extraction of Bactericidal Substances from Polymorphonuclear Leucocytes.—

The standard procedure consisted, in essence, of disruption of the phagocytic cells by alternate freezing and thawing, and extraction with a salt solution (ICS) made to imitate the cationic composition of rabbit microphagocytic cytoplasm.

Various methods for cell disruption were investigated, and all yielded approximately the same results. These included three cycles of freezing at −75°C. and thawing at 38°C., ten cycles of similar freezing and thawing, mechanical grinding, and sonic oscillation. Loss of bactericidal activity occurred when leucocyte suspensions were subjected to sonic oscillation for periods longer than 2 minutes.

Studies were also done to determine whether more bactericidal activity might be extracted with solvents other than ICS solution. The following solvents were all found to be equally efficient in this regard: ICS solution, 0.9 per cent saline, 10 per cent saline, 0.1 M sodium citrate-HCl pH 4.0, 0.1 M sodium citrate-HCl pH 6.0, 0.1 M mixed phosphate buffer pH 7.4, 0.1 M mixed phosphate buffer pH 8.5, ICS solution containing 2 per cent triton WR 1339, ICS solution containing 50 per cent glycerol, and 5 per cent bovine albumin in 0.9 per cent saline.

The Range of the Bactericidal Action of Rabbit Leucocyte Extracts.—

The phagocyte extract used in these tests was derived from a pool of polymorphonuclear leucocytes (7.7×10^9 cells) obtained from 7 rabbits. The leucocyte buttons were suspended in 15.4 ml. of 0.9 per cent saline and subjected to three cycles of freezing and thawing. After centrifugation, the supernatant was distributed in several pyrex tubes and maintained at −21°C. until used in the bactericidal tests.

The microorganisms were, except as otherwise noted below, from a stock culture collection maintained for many months in artificial media. All were grown in beef heart-peptone broth except the group C streptococcus which was cultivated in broth medium enriched with 10 per cent defibrinated rabbit blood, and the mycobacteria which were maintained in tween albumin liquid medium. The strain of *Escherichia coli* B was kindly supplied by Dr. Guy Barry of The Rockefeller Institute. The *Proteus vulgaris* and *Proteus mirabilis* were obtained through the courtesy of Miss Leask of New York Hospital; they were strains freshly isolated from human urine specimens.

Bactericidal tests on many of the Gram-positive bacteria were unsatisfactory in ICS medium, since some of them perished in this environment even when no leucocyte extract was added. Duplicate bactericidal tests were therefore done in a medium composed of 0.05 M acetate-phosphate buffer at pH 6.0 containing a final concentration of 0.2 per cent glucose and 0.05 per cent bovine plasma albumin (AGAP medium). As will be shown later, the bactericidal activity of leucocyte extracts on Gram-negative enteric bacilli was approximately the same in ICS and in AGAP media.

As is shown in Table III, many Gram-negative enteric bacilli, including *Pseudomonas*, *Escherichia*, *Shigella*, *Salmonella*, *Klebsiella*, and *Proteus* types, were killed by the phagocyte extracts but not by lysozyme under these conditions. Two *Proteus* strains freshly isolated from human urine were unaffected by similar exposure. *Escherichia coli* 28B2, kindly supplied by Dr. Pillemer's laboratory, was a strain known to be resistant to killing by the properdin system. Several strains of Gram-negative bacteria, not otherwise characterized and not listed in the table, were isolated from rabbit feces. These microbes manifested susceptibility to the lethal action of rabbit phagocyte extracts similar to that shown for *Shigella sonnei* in Table III.

Among the Gram-positive microorganisms examined, only the aerobic spore former *Bacillus megatherium* was susceptible to the bactericidal action of the leucocyte extracts. Staphylococci, streptococci, and mycobacteria were resistant to this bactericidal action under the conditions of the test.

TABLE III

Bactericidal Activity of Leucocyte Extracts

Dilution of phagocyte extract in ICS solution	Percentage of bacteria killed in 1 hr. at 38°C.							
	Pseudomonas aeruginosa	*Escherichia coli* B	*Escherichia coli* 28B2	*Shigella sonnei*	*Salmonella enteritidis*	*Klebsiella pneumoniae* C	*Proteus* 2	*Bacillus* megatherium*
1:2	100	100	100	100	99	100	100	100
1:4	100	100	100	100	91	100	100	100
1:8	100	100	100	100	91	99	100	100
1:16	100	100	100	100	51	91	100	100
1:32	93	100	100	85	0	0	90	100
1:64	66	100	0	0	0	0	0	100
1:128	0	100	0	0	0	0	0	100
1:256	0	100	0	0	0	0	0	98
1:512	0	97	0	0	0	0	0	98
1:1024	0	90	0	0	0	0	0	80
1:2048	0	0	0	0	0	0	0	0
Lysozyme 1 mg./ml.	0	0	0	0	0	0	0	—

Dilution of phagocyte extract in ICS solution	Percentage of bacteria killed in 1 hr. at 38°C.							
	Proteus vulgaris	*Proteus mirabilis*	*Micrococcus* pyogenes,* var. *albus*	*Micrococcus* pyogenes,* var. *aureus*	*Streptococcus** H69D	*Streptococcus* hemolyticus* C	*Mycobacterium phlei*	*Mycobacterium tuberculosis* (BCG)
1:2	0	0	0	0	0	0	0	0

* Bactericidal tests on these microorganisms were performed in AGAP medium (containing albumin, glucose, acetate, and phosphate) (see text).

These results represent only preliminary studies of the range of bacteria affected by phagocyte extracts. Complete investigation of this point has been postponed until the tests can be conducted with isolated, or at least purified, preparations of the bactericidal substances(s) concerned.

Evidence That the Action of Phagocyte Extracts on Susceptible Bacteria Was Lethal But not Lytic.—

Approximately 1.9×10^9 polymorphonuclear leucocytes were suspended in 10 ml. of 0.1 M sodium citrate adjusted to pH 5.6 by the addition of 1 N HCl. After disruption and centrifugation, the supernatant was dialyzed against several large volumes of the same citrate-HCl buffer (no activity was lost on dialysis as will be discussed in a later section). An overnight culture of *Shigella sonnei* was divided into 3 equal portions and the bacteria were washed twice with citrate-HCl buffer pH 5.6 by centrifugation. One bacterial button was suspended in the dialyzed leucocyte extract, one in buffer alone, and the third in a solution of lysozyme in citrate-HCl buffer. The optical density of each was read promptly in a Coleman, Jr., spectrophotometer at 650λ. After incubation at 38°C. for 90 minutes, optical densities were again

recorded. The bacteria in the specimens were then washed three times in the centrifuge with citrate-HCl buffer, and serial 10-fold dilutions of the final suspension were plated on beef heart-peptone agar to determine the numbers of surviving bacteria.

TABLE IV

Demonstration That the Action of Rabbit Polymorphonuclear Leucocyte Extracts on Shigella sonnei Was Bactericidal but Not Bacteriolytic

Shigella sonnei suspended in	Optical density at time of mixing	Optical density after incubation for 90 min. at 38°C.	No. of surviving bacteria per 0.1 ml. after 90 min. at 38°C.
Phagocyte extract dialyzed against sodium citrate-HCl buffer pH 5.6	0.142	0.141	0
Sodium citrate-HCl buffer pH 5.6	0.062	0.062	3.2×10^7
0.1 per cent lysozyme in sodium citrate-HCl buffer pH 5.6	0.062	0.061	2.6×10^7

As is seen in Table IV, the dialyzed extract of rabbit polymorphonuclear leucocytes did not detectably lyse *Shigella sonnei* under these conditions. The higher initial and final optical density of the bacteria suspended in phagocyte extract as compared to those in buffer alone was due to the opalescent character of the extract. Smears of the bacterial suspensions made promptly, after incubation and after washing all showed typical small Gram-negative rods; the *Shigella sonnei* which had been incubated with the leucocyte extract were not clumped and the morphology of the individual bacterial cells was not notably different from that of cells from the control or lysozyme suspensions. That the microorganisms were killed by the leucocyte extract was indicated by their failure to grow on suitable culture media even after they had been washed thoroughly. The results in the table also show that lysozyme exerted no lethal or lytic effect on *Shigella sonnei.*

The Influence of the Age of the Bacteria on Their Susceptibility to the Lethal Effect of Leucocyte Extracts.—

In several experiments on *Escherichia coli* B and on *Shigella sonnei*, cultures ranging from 2 hours to 1 week old showed no differences in susceptibility to the bactericidal action of phagocyte extracts.

The Bactericidal Activity of Extracts of Various Rabbit Organs and Tissues as Compared to That of Rabbit Polymorphonuclear Leucocytes.—

It was of interest to determine whether the bactericidal activity was peculiar to the polymorphonuclear leucocyte or was generally distributed in many rabbit tissue cells.

After a peritoneal exudate was collected in standard fashion, the rabbit was sacrificed by exsanguination. The entire spleen, 1 lobe of the liver, the entire heart, 1 kidney, and approximately 10 gm. of skeletal muscle were then removed. The amount of each of the organ speci-

mens was at least ten times greater than that of the leucocyte button. The organs were individually minced with scissors and the resulting small blocks of tissue were washed two times by centrifugation in citrate saline to remove blood and other extracellular fluids. The packed tissue cells and the leucocyte button were then each suspended in 5 ml. of ICS solution, the cells broken by alternate freezing and thawing, and the supernatants collected in the centrifuge.

TABLE V

Bactericidal Activity of Extracts of Various Rabbit Organs and Tissues

Dilution of organ extract in ICS medium	Percentage of *Klebsiella pneumoniae* type C killed					
	PMN leucocyte	Spleen	Liver	Heart	Kidney	Skeletal muscle
1:2	99	0	0	0	0	0
1:20	0	0	0	0	0	0
Dilution of organ extract in ICS medium	Percentage of *Escherichia coli* B killed					
	PMN leucocyte	Spleen	Liver	Heart	Kidney	Skeletal muscle
1:2	100	100	100	0	0	0
1:20	100	100	100	0	0	0
1:200	90	90	0	0	0	0
1:2000	80	0	0	0	0	0

As is seen in Table V, even though much larger quantities of the various tissues than of leucocytes were used, bactericidal activity for *Klebsiella pneumoniae* type C was manifested only in the leucocyte extract. When tests were performed with the more susceptible *Escherichia coli* B, some bactericidal activity was demonstrated in the spleen and liver extracts, but none was found in those of heart, kidney, or skeletal muscle. The activity in the preparations of spleen and liver was quantitatively much less than that of the leucocyte extract. Since the spleen and liver are sites of white cell formation and collection, their bactericidal activity may have been derived from the polymorphonuclear phagocytes contained therein.

The Bactericidal Activity of Extracts of Polymorphonuclear Leucocytes from Animals Other than the Rabbit.—

The human leucocytes were obtained with the kind cooperation of Dr. David Rogers of New York Hospital. They were collected from a patient who was suffering from "sterile" empyema complicating pneumococcal infection. A thoracentesis was performed and 700 ml. of a thick brown fluid removed. The material was processed promptly, using the same technique as that developed for rabbit leucocytes. (It is of interest that the human leucocyte button after red cell lysis and washing was yellowish green in color, in contrast to the pearly white appearance of rabbit cells.) Over 95 per cent of the cells were polymorphonuclear, and supravital staining with trypan blue revealed over 90 per cent to be viable.

Guinea pig polymorphonuclear phagocytes were obtained from 5 young adult albino

animals. Each pig was injected intraperitoneally with 50 ml. of saline containing glycogen. 4 hours later the animals were sacrificed and the exudates collected and pooled. Approximately 10^9 leucocytes were obtained from the 5 pigs. Since these exudates appeared to be bloodless, the step of exposure to "lysis solution" was omitted from their processing, which was otherwise identical to that employed for rabbit cells. The guinea pig leucocytes were over 90 per cent polymorphonuclear ones, and staining with trypan blue showed that over 80 per cent were viable.

Exudates were also collected from adult Sherman-Wistar rats using a technique identical to that described for guinea pigs immediately above. Polymorphonuclear leucocytes were obtained from Rockefeller Swiss mice in similar fashion except that each mouse was injected with 5 ml. of saline containing glycogen. The exudate cells from these rodents were predominantly neutrophilic microphages.

TABLE VI

Bactericidal Activity of Extracts of Human and Guinea Pig Polymorphonuclear Leucocytes

Cell extract and dilution in ICS medium		Percentage of bacteria killed in 1 hr. at 38°C.		
		Escherichia coli B	*Shigella sonnei*	*Klebsiella pneumoniae* C
Human	1:2	100	0	0
	1:4	100	0	0
	1:8	100	0	0
	1:16	100	0	0
	1:32	98	0	0
	1:64	100	0	0
	1:128	98	0	0
	1:256	96	0	0
	1:512	0	0	0
Guinea pig	1:2	100	0	0
	1:4	100	0	0
	1:8	100	0	0
	1:16	98	0	0
	1:32	92	0	0
	1:64	60	0	0
	1:128	0	0	0

As is shown in Table VI, extracts of human and of guinea pig polymorphonuclear leucocytes manifested bactericidal activity on *Escherichia coli* B which was quantitatively much less than that of rabbit white cells. Moreover, they did not kill *Shigella sonnei* or *Klebsiella pneumoniae*. Extracts of rat and of mouse polymorphonuclear leucocytes exerted no detectable lethal effect on *Escherichia coli* B.

Differentiation of the Bactericidal Activity of Rabbit Leucocyte Extracts from That of the Properdin System.—

Certain features of the bactericidal activity of rabbit leucocyte extracts which suggested that it was unrelated to the properdin system are described

in an accompanying communication (11). These features were: (*a*) the enhanced activity of phagocyte extracts at an acid pH, and (*b*) the absence of a requirement for complement or magnesium ions. To determine with certainty whether or not components of the properdin system were present, rabbit white cells were processed in a special manner so that extracts of them would be suitable for properdin and complement assay. Dr. Louis Pillemer kindly performed these assays.

TABLE VII

Bactericidal Activity of Rabbit Polymorphonuclear Extracts Free of Properdin and Complement

Escherichia coli B			*Shigella sonnei*			*Klebsiella pneumoniae* C		
Phagocyte extract, dilution	Medium	Per cent killed in 1 hr. at 38°C.	Phagocyte extract, dilution	Medium	Per cent killed in 1 hr. at 38°C.	Phagocyte extract, dilution	Medium	Per cent killed in 1 hr. at 38°C.
A 11/10 1:100	ICS	99	A 11/10 1:10	ICS	98	A 11/10 1:4	ICS	100
1:200	"	98	1:20	"	80	1:8	"	97
1:400	"	83	1:40	"	62	1:16	"	80
1:800	"	0	1:80	"	0	1:32	"	0
1:1600	"	0	1:160	"	0	1:64	"	0
A 11/10 1:100	AGAP*	98	A 11/10 1:10	AGAP*	100	A 11/10 1:4	AGAP*	98
1:200	"	83	1:20	"	99	1:8	"	99
1:400	"	0	1:40	"	94	1:16	"	98
1:800	"	0	1:80	"	88	1:32	"	88
1:1600	"	0	1:160	"	0	1:64	"	66
B 11/10 1:100	ICS	100	B 11/10 1:10	ICS	94	B 11/10 1:4	ICS	100
1:200	"	100	1:20	"	78	1:8	"	99
1:400	"	79	1:40	"	0	1:16	"	98
1:800	"	0	1:80	"	0	1:32	"	75
1:1600	"	0	1:160	"	0	1:64	"	0
B 11/10 1:100	AGAP*	95	B 11/10 1:10	AGAP*	100	B 11/10 1:4	AGAP*	100
1:200	"	94	1:20	"	97	1:8	"	100
1:400	"	91	1:40	"	77	1:16	"	93
1:800	"	76	1:80	"	0	1:32	"	82
1:1600	"	0	1:160	"	0	1:64	"	0

* Medium containing albumin, glucose, acetate, and phosphate. See text for full description.

Exudates were induced in 2 rabbits in the usual fashion. The cells were collected from one (A 11/10) by peritoneal lavage with physiological saline rather than with citrate saline. After standing at 6°C. for 30 minutes, the flask was agitated vigorously and the fibrin clot which had formed was removed by filtration through gauze. The resulting filtrate contained 1.1×10^9 leucocytes and was only lightly contaminated with red cells. After washing three times in the centrifuge with 0.9 per cent NaCl, the cells were suspended in 10 ml. of physiological saline and were disrupted in the standard manner. The supernatant was distributed in chemically

clean pyrex tubes and some of these were stored and shipped to Dr. Pillemer in dry ice. This cell preparation thus differed from the standard one in that there was at no time exposure to citrate or other metal binding agents. The second rabbit (B 11/10) yielded 2.2×10^9 leucocytes, and this exudate was collected and processed in the standard manner, including lysis of red cells, except that the final cell buttom was washed once with 0.9 per cent saline in order to eliminate the citrate trapped therein.

These cell extracts were examined by Dr. Pillemer, and were also tested for bactericidal activity by the present author. Tests were done in ICS solution, and also in a medium containing albumin, glucose, acetate, and phosphate (AGAP) which has been described in a preceding section.

Dr. Pillemer could detect no properdin, complement, anti-properdin activity, or anticomplementary substances in these leucocyte extracts. Table VII presents their bactericidal activity on enteric bacilli in ICS solution and in an acetate-phosphate buffer containing glucose and albumin (AGAP). It is seen that striking bactericidal activity was manifested in both of these media.

STUDIES OF THE CHEMICAL NATURE OF THE SUBSTANCE RESPONSIBLE FOR BACTERICIDAL ACTIVITY OF LEUCOCYTE EXTRACTS ON ENTERIC BACILLI

When leucocyte extracts were dialyzed against saline, ICS solution, 0.1 M citrate-HCl pH 5.6, or 0.1 M phosphate buffer pH 7.4, all bactericidal activity remained inside the cellophane bag. Dialysis against distilled water resulted in the formation of a precipitate and in considerable loss of bactericidal activity from the supernatant. This activity could not be recovered completely by dissolving the precipitate in salt solutions.

TABLE VIII

Destruction of the Bactericidal Activity of Leucocyte Extracts by Trypsin

Dilution of samples at right in 0.1 M sodium citrate-HCl pH 5.0	Percentage of *Escherichia coli* B killed in 1 hr. at 38°C. by diluted phagocyte extracts incubated in 0.1 M mixed phosphate buffer pH 8.5 for					
	1 min. at		90 min. at			
	0°C.		0°C.		38°C.	
	No trypsin	0.5 mg./ml. salt free crystallized trypsin	No trypsin	0.5 mg./ml. salt-free crystallized trypsin	No trypsin	0.5 mg./ml. salt-free crystallized trypsin
1:10	100	100	100	100	100	88
1:20	100	100	100	56	100	0
1:40	0	0	0	0	0	0

The data in Table VIII demonstrate that leucocyte extracts incubated for 90 minutes with crystallized trypsin lost a part of their bactericidal activity for *Escherichia coli* B. The findings were similar when *Shigella sonnei* or *Klebsiella pneumoniae* were used as test organisms. The antimicrobial action of phagocyte extracts disappeared rapidly on incubation with mixed pancreatic enzymes, but

no detectable change resulted from exposure to crystalline deoxyribonuclease or streptococcal nucleolytic enzymes.

Fractionation of Polymorphonuclear Leucocyte Extracts.—

The results of fractionation of leucocyte extracts with ammonium sulfate are presented in Table IX.

2×10^9 leucocytes were extracted by freezing and thawing in 25 ml. of 0.1 M sodium citrate adjusted to pH 5.6 with 1 N HCl. After centrifugation the supernatant was dialyzed overnight at 4°C. against a large volume of citrate-HCl pH 5.6. 5 ml. of this dialyzed extract was stored at 4°C. ("starting cell extract"). The remaining 20 ml. was fractionated by salting out with ammonium sulfate, which was introduced from saturated solutions by dialysis with agitation overnight at 4°C. Precipitates which formed at the concentration levels indicated in the table were collected in the centrifuge. After each step the supernatants were adjusted to 20 ml. by adding ammonium sulfate solutions of appropriate concentration. Solutions of each of the precipitates in 20 ml. of citrate-HCl buffer pH 5.6, and the supernatant at 80 per cent saturation with ammonium sulfate were dialyzed against several changes of citrate-HCl buffer to rid them of ammonium sulfate.

Bactericidal activity of these fractions on *Shigella sonnei* was determined in standard fashion except that the tests were done in citrate-HCl buffer pH 5.6 rather than in ICS solution.

TABLE IX

Ammonium Sulfate Fractionation of Leucocyte Extracts

Dilution of fractions of phagocyte extracts in citrate-HCl buffer pH 5.6	Percentage of *Shigella sonnei* killed in 1 hr. at 38°C. by				
	Starting cell extract	Fraction precipitated at 40 per cent saturation with $(NH_4)_2SO_4$	Fraction soluble at 40 per cent and precipitated at 60 per cent saturation with $(NH_4)_2SO_4$	Fraction soluble at 60 per cent and precipitated at 80 per cent saturation with $(NH_4)_2SO_4$	Fraction soluble at 80 per cent saturation with $(NH_4)_2SO_4$
Whole	100	0	100	0	0
1:2	100	0	97	0	0
1:4	97	0	100	0	0
1:8	100	0	82	0	0
1:16	88	0	64	0	0
1:32	0	0	0	0	0
	Approximate lysozyme content of above fractions, *mg./ml.**				
	0.01	0.0005	0.005	0.005	0.001

* As determined by comparison with crystalline egg white lysozyme in their capacity to lyse *M. lysodeikticus* in a beef heart–peptone medium at pH 7.4.

As is shown in Table IX, the fraction soluble at 40 per cent and precipitated at 60 per cent saturation with ammonium sulfate was the only one manifesting bactericidal activity on *Shigella sonnei*. Moreover, this fraction was quantita-

tively as active as the whole cell extract, and its activity was not enhanced when the other fractions were combined with it in a pool. In contrast, most of the lysozyme activity was equally distributed between fractions precipitated at 60 per cent and at 80 per cent saturation with salt.

Cold ethanol fractionation of the leucocyte extracts was also attempted, with results shown in Table X.

9×10^8 leucocytes were extracted in 25 ml. of ICS solution. A 5 ml. portion of this extract was removed and maintained at 4°C. ("starting cell extract"). With constant stirring and with all reagents at 4°C., 95 per cent ethanol was added dropwise to the remaining 20 ml. of extract, and fractions were collected by centrifuging in the cold. The precipitates were dissolved in 20 ml. of ICS solution, and were dialyzed against several large volumes of this medium to remove residual ethanol.

TABLE X

Cold Ethanol Fractionation of Leucocyte Extracts

Dilution of fractions of phagocyte extracts in ICS medium	Percentage of *Escherichia coli* B killed in 1 hr. at 38°C. by			
	Starting cell extract	Fraction precipitated at 32 per cent ethanol concentration	Fraction soluble at 32 per cent and precipitated at 45 per cent ethanol concentration	Fraction soluble at 45 per cent and precipitated at 80 per cent ethanol concentration
1:10	100	100	100	0
1:20	100	100	78	0
1:40	100	80	0	0
1:80	100	70	0	0
1:160	100	0	0	0
1:320	100	0	0	0
1:640	0	0	0	0
	Approximate lysozyme content of above fractions* *mg./ml.*			
	0.01	<0.005	<0.005	0.01

* As determined by comparison with crystalline egg white lysozyme in their capacity to lyse *M. lysodeikticus* in mixed phosphate buffer pH 7.4.

As is seen in Table X, material manifesting a bactericidal effect on *Escherichia coli* B was recovered predominantly in the fraction precipitated at 32 per cent ethanol concentration. A moderate over-all loss of activity occurred during ethanol fractionation by this relatively crude technique. Of particular interest was the dissociation of lysozyme activity and of bactericidal activity on *Escherichia coli* B. All of the lysozyme-like material was found in the 80 per cent ethanol fraction, a fraction which manifested no lethal effect on the coliform microorganisms.

Leucocyte extracts were also fractionated in the ultracentrifuge.

In this experiment 10^9 leucocytes were suspended in 4 ml. of 5 per cent bovine albumin in 0.9 per cent saline. After three cycles of freezing and thawing, the supernatant was collected and dialyzed against 500 ml. of physiological saline with constant agitation at 4°C. for 4 hours. An aliquot was retained as a control, and the remainder was divided into three portions which were adjusted to densities indicated in Table XI by addition of solutions of sodium nitrate and potassium bromide. Dr. Rhodes Trautman kindly centrifuged these specimens (Spinco model E 85, 27°C., 36,000 to 37,000 R.P.M. for 18 hours). Each was then separated into top and bottom fractions. Gross light scattering observation suggested that of the top fractions, only the one at density 1.20 contained significant amounts of macromolecular material.

In testing the bactericidal activity, a starting dilution of 1:100 was made in ICS solution to minimize possible effect of the protein and salts in the fractions.

TABLE XI

Fractionation of Leucocyte Extracts in the Ultracentrifuge

Dilution of fractions of phagocyte extract in ICS medium	Percentage of *Escherichia coli* B killed in 1 hr. at 38°C. by fractions of cell extracts subjected to ultracentrifugation in a medium of density						Control sample (not centrifuged)
	1.01		1.06		1.20		
	Top fraction	Bottom fraction	Top fraction	Bottom fraction	Top fraction	Bottom fraction	
1:100	0	100	0	100	0	100	100
1:200	0	98	0	100	0	100	100
1:400	0	100	0	100	0	100	100
1:800	0	98	0	98	0	98	100
1:1600	0	80	0	74	0	82	90
1:3200	0	0	0	0	0	0	0

As is revealed by the data in Table XI, only the bottom fractions manifested a lethal effect on *Escherichia coli* B, regardless of the density of the solutions during centrifugation. There was no detectable loss of activity as a result of this operation. Also, pools of top and bottom fractions (not shown in the table) exhibited an antimicrobial effect corresponding to that of the bottom fraction alone. These results thus suggested that the bactericidal material was not a lipoprotein.

That the substance responsible for bactericidal activity of rabbit leucocyte extracts was, at least in part, a protein was thus supported by several findings: its failure to pass through cellophane membranes, its inactivation by crystallized trypsin, and its precipitation and quantitative recovery on ammonium sulfate fractionation. The microbiological and chemical observations also supported the conclusion that this substance differed from previously characterized antibacterial agents from tissues. It has therefore been called phagocytin, a name which connotes both its origin and its protein nature.

The Stability of Phagocytin.—

The results of studies on the heat stability of phagocytin are presented in Table XII.

For this experiment 8×10^8 polymorphonuclear leucocytes were frozen and thawed in 10 ml. of physiological saline. After centrifugation, the supernatant was mixed with 10 ml. of ICS solution. This mixture was divided into small portions and held under the conditions outlined in the table.

TABLE XII

Heat Stability of Phagocytin

Dilution of phagocyte extracts in ICS medium after exposure to conditions at right	Percentage of *Escherichia coli* B killed in 1 hr. at 38°C. by cell extracts held for															
	30 min. at						2 hrs. at					20 hrs. at				
	−21°C.	38°C.	46°C.	56°C.	65°C.	100°C.	−21°C.	38°C.	46°C.	56°C.	65°C.	−21°C.	38°C.	46°C.	56°C.	65°C.
Whole	100	100	100	100	100	0	100	100	100	100	100	100	100	100	100	100
1:10	100	100	100	100	100	0	100	100	100	98	100	100	98	100	100	100
1:20	100	100	100	100	100	0	100	100	98	100	98	100	100	100	100	85
1:40	100	100	100	100	100	0	100	98	78	94	72	100	100	94	100	55
1:80	100	100	100	100	100	0	92	94	0	0	0	100	100	0	0	0
1:160	50	92	60	96	84	0	60	80	0	0	0	85	0	0	0	0
1:320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

The capacity of white cell extracts to kill *Escherichia coli* B was lost after heating to 100°C. for 30 minutes. At −21°C. and at 38°C., there was no significant change in activity during 20 hours of exposure. And, at 46°C., 56°C., and 65°C., only slight inactivation occurred.

It was found, however, that the bactericidal capacity of phagocyte extracts diminished on prolonged storage in the cold. The results of a representative experiment dealing with this point are presented in Table XIII.

A precipitate commonly appeared in white cell extracts on storage. It was reasoned that this delayed precipitation might be due to lipoproteins. Even though the ultracentrifuge studies indicated that phagocytin was not a lipoprotein, it seemed possible that these substances might bring about loss of activity by adsorbing phagocytin. Therefore various agents (rutin, ethylenediaminetetraacetic acid, tocopherol, NaCl) known to stabilize lipoproteins were added to some samples of leucocyte extracts to determine whether they might confer stability on phagocytin.

As is seen in Table XIII, roughly nine-tenths of the bactericidal activity on *Escherichia coli* B was lost in leucocyte extracts after standing at 4°C. for a period of 3 weeks. None of the agents commonly used to stabilize lipoproteins

TABLE XIII

Lack of Stability of the Bactericidal Activity of Leucocyte Extracts on Standing for Prolonged Periods of Time at 4°C.

Cell preparation and extracting medium	Highest 2-fold dilution of cell extract producing killing of over 50 per cent of *Escherichia coli* B in 1 hr. at 38°C. of cell extracts standing at 4°C. for		
	20 hrs.	5 days	22 days
I 10/11 in ICS solution	1:600	1:100	1:60
" " ICS-saturated with rutin	1:800	1:400	1:160
" " ICS-0.1% EDTA-Na2*	1:800	1:200	1:60
" " ICS-saturated with α-tocopherol phosphate	1:800	1:400	1:20
K 11/10 in ICS	1:3200	1:1600	1:400
" " 10 per cent NaCl pH 6.0	1:6400	1:1600	1:50
" " " " " " " 7.0	1:6400	1:3200	1:800
" " " " " " " 8.0	1:6400	1:3200	1:800

* Disodium salt of ethylenediaminetetraacetic acid.

had any detectable effect on the stability of phagocytin. Further studies, not recorded in the table, indicated that phagocytin was reasonably stable on storage at −21°C. for periods of at least 2 months.

Studies were also done dealing with the short term stability of phagocytin as related to the reaction of the medium. Stability was approximately the same in ICS solution, in ICS solution adjusted to pH 4.5 by the addition of 1 N HCl, in 0.1 M mixed phosphate buffer pH 8.5, and in 0.1 M dibasic sodium phosphate adjusted to pH 11.0 by the addition of 1 N NaOH.

Solutions of phagocytin in 5 per cent bovine albumin (see procedure for ultracentrifuge fractionation above) appeared to be perfectly stable on storage for 2 months at 4°C. Also, preliminary studies suggested that cell extracts which had been dialyzed against citrate buffer at pH 5.6 retained their bactericidal activity on standing in the cold. The factors responsible for the variable stability of phagocytin are at present unknown.

The Inactivation, Presumably by Adsorption, of Phagocytin by Various Materials.—

Bactericidal activity disappeared from extracts of polymorphonuclear leucocytes after exposure to a variety of materials. The results of an experiment demonstrating this phenomenon are shown in Table XIV.

The adsorbents listed in the table were used in 100 mg. quantities except for zymosan (10 mg., Standard Brands, Inc., New York) and for the bacteria (10^9 bacterial cells). The

microorganisms were killed by heating at 100°C. for 15 minutes. The adsorbents were washed twice in the centrifuge with ICS solution or with 0.02 M phosphate buffer pH 8.0, and were then mixed with 1 ml. quantities of leucocyte extracts in these same buffers. After incubation with occasional agitation for 1 hour at 38°C., they were centrifuged and the bactericidal activity of the supernatants was tested in standard fashion.

TABLE XIV

Inactivation, Presumably by Adsorption, of Phagocytin by Various Materials

Dilution of phagocyte extract in ICS medium	Percentage of *Escherichia coli* B killed in 1 hr. at 38°C. by phagocyte extract which had been exposed to							
	Al_2O_3	$BaSO_4$	Potato starch	Denatured casein	Zymosan	Heat-killed *Escherichia coli* B	Heat-killed *Micrococcus pyogenes* var. *aureus*	None
1:5	0	0	100	0	0	0	0	100
1:25	0	0	99	0	0	0	0	98

As is shown in Table XIV, bactericidal activity on *Escherichia coli* B disappeared after exposure of leucocyte extracts to aluminum oxide, barium sulfate, acid denatured casein, zymosan, or heat-killed bacteria. Staphylococci, which were not killed by leucocyte extracts, removed activity as did the susceptible coliform microorganisms. Potato starch was the only material which did not inactivate phagocytin. The results presented in Table XIV were the same whether the exposure to adsorbents took place in ICS solution or in phosphate buffer at pH 8.0. Further studies, not included in the table, showed that phagocytin was also inactivated by filter paper, porcelain bacterial filters, cation exchange resins, and calcium phosphate.

It was assumed that these materials adsorbed phagocytin. Therefore attempts were made to elute bactericidal activity. In no instance did this prove possible, even when strong salt solutions or alkaline reactions were employed. Combinations of unadsorbed material in the supernatants and eluates from the adsorbents were likewise inactive.

DISCUSSION

Several studies of the bactericidal activity of leucocyte extracts were conducted around 1900. At that time there was considerable debate as to whether the serum bactericidal agent (alexin or complement) was derived from leucocytes as postulated by Metchnikoff (2). Schattenfroh (12), Pettersson (13), Schneider (14), and Kling (15) all found that extracts of exudate cells from various animals killed certain bacteria *in vitro*, and demonstrated that this white cell material differed in heat stability and in bactericidal activity from the bactericidal substance in serum. Hiss (16) and Hiss and Zinsser (17)

administered rabbit leucocyte extracts to experimental animals and to human beings suffering from various infectious diseases and were impressed that beneficial effects resulted. Except for a report by Haussmann (18) in 1925, little further work has been done to confirm the presence of bactericidal agents other than lysozyme in leucocytes. Although these early studies do not present sufficient data to permit comparison to the material characterized in the present communication, they may well be similar or identical.

Although the range of bacteria susceptible to phagocytin has not been completely studied, this material seems to act most strikingly on Gram-negative enteric bacilli. Various coliform microorganisms maintained on artificial media for long periods of time were all found to be killed by leucocyte extracts, while two strains of *Proteus* recovered from human urine were unaffected. Whether the resistance of these *Proteus* strains to phagocytin is an inherent property, or whether it might be related to their recent isolation from an animal source remains to be determined. It is of interest that in 1905 Pettersson found wide differences in the susceptibility of numerous *Proteus* strains to killing by leucocyte extracts (13).

Certain bacteria, for example staphylococci of the *albus* type, are known to be killed in the living polymorphonuclear phagocyte (19), yet manifest no susceptibility to leucocyte extracts as tested in these experiments. It is still possible, however, that phagocytin plays a role in destroying such microorganisms *in vivo*. The preparations of phagocytin represent considerable dilution of intracellular constituents compared to the situation in the intact cell. It might be pointed out that staphylococci were killed on incubation with disrupted but undiluted leucocyte buttons (see first section in Results). On the other hand, it is of course possible that Gram-positive cocci are destroyed inside phagocytes by a biochemical mechanism unrelated to that which kills Gram-negative enteric bacteria.

At the outset of this study, it was reasoned that there might be two broad mechanisms by which phagocytes kill bacteria. First, perhaps they contained in their cytoplasm a preformed bactericidal agent; phagocytin may well be such a material. The other possible mechanism was that bactericidal conditions might develop within phagocytes only as a consequence of engulfment of a foreign particle. This second mechanism might be thought of in terms of "intracellular inflammation." For instance, perhaps phagocytes respond to the ingestion of certain parasites by producing antimicrobial intracellular concentrations of lactic and other organic acids characteristic of the inflammatory reaction. In a sense the phagocytes might thus perform an important function by sequestering the microbes. Obviously tissues in general cannot develop a high degree of acidity for this would result in death of the host. However, even if intraphagocytic acidity should, as may often be the case, result in death of the leucocyte, the host survives since phagocytic cells are expendable. Perhaps,

then, a mechanism of this general type may be responsible for death of those bacteria susceptible to phagocytic killing but resistant to phagocytin.

The observations made thus far suggest that phagocytin may exert a bactericidal action *in vivo* as it does *in vitro*. Since leucocytes simply disrupted by freezing and thawing or extracted in saline manifest activity, it seems unlikely that microphagocytic cytoplasm contains substances or conditions which neutralize the bactericidal effect.

Phagocytin is probably located in the cytoplasm of the polymorphonuclear cells. In some of the disruption and extraction procedures employed, nuclear elements and cytoplasmic granules remained particulate. After centrifugation, bactericidal activity was found in the soluble fraction, and it was not possible to liberate additional activity by disruption and further extraction of the particulate elements.

Since phagocytin exerts a bactericidal but not a bacteriolytic effect, it does not disrupt the bacterial cell wall in the gross. The absence of lysis does not, however, preclude an action primarily at the surface; some surface-active bactericidal agents kill rapidly without lysing the microorganisms (20).

Human and guinea pig polymorphonuclear leucocytes yielded only small amounts of material manifesting a lethal action on coliform bacteria, and none was obtained from rat and mouse phagocytes. Perhaps the nature of the bactericidal substance in white cells and the conditions required for its action differ among species of animals. Also, it might be pointed out that phagocytin is destroyed by proteolytic enzymes, and it has been previously shown that among polymorphonuclear phagocytes from various mammals, only rabbit cells contain no trypsin-like leucoprotease (21). Therefore phagocytin might be obtained with relative ease from rabbit leucocytes simply because they have no proteolytic enzyme which will attack it.

Although conclusive demonstration that phagocytin activity is due to a single substance must await its isolation, the available evidence suggests that this may well be the case. In the various manipulations of the cell extracts, bactericidal activity on enteric bacilli was always found in a single or in closely related fractions, and in no instance was activity enhanced by combining the various fractions.

The lack of stability of phagocytin on prolonged storage in the refrigerator is at present unexplained. It might be due to: (*a*) inherent instability of the protein or of some unrecognized other constituent necessary for its action, (*b*) removal of phagocytin by adsorption to other materials, perhaps lipoproteins, which become insoluble on standing, or (*c*) slow destruction of phagocytin by enzymes in the cell extracts.

The apparent ease with which phagocytin is adsorbed by a variety of substances may be fairly non-specific, for this behavior is characteristic of many proteins in dilute solution. Since phagocytin is bound to both susceptible and

to apparently resistant killed bacterial cells, it seems unlikely that adsorption is of prime importance in determining which microorganisms are susceptible to its action.

It is important to emphasize that the studies in this report deal with but one type of phagocytic cell, the polymorphonuclear leucocyte. The mechanisms which destroy parasites within mononuclear phagocytes are completely unknown. These mechanisms may be quite different from those in the microphages; for instance, although large amounts of lysozyme are found in polymorphonuclear leucocytes, essentially none of this antibacterial enzyme is detectable in macrophages (6).

The technique presented here which permits collection of large numbers of polymorphonuclear leucocytes in a living "biologically pure" state should be of value for investigation of intracellular physiology and pathology in general. Except for isolated components or reactions (10), knowledge concerning the biochemistry of cytoplasm is meager. Studies of intracellular phenomena in organ cells are complicated by the difficulty of isolating them intact and free from extracellular fluids. With phagocytic cells this difficulty may be readily circumvented.

SUMMARY

A technique has been developed for collecting large numbers of polymorphonuclear leucocytes from peritoneal exudates in rabbits. These cells are obtained essentially free from other cell types and from debris.

When microphages so procured are disrupted by physical methods and extracted with aqueous salt solutions, the soluble fraction manifests striking bactericidal activity, especially on Gram-negative enteric bacilli. The susceptible microorganisms are not lysed.

This bactericidal substance, which has been called phagocytin, appears to be limited in distribution mainly to the polymorphonuclear leucocyte. No phagocytin is present in extracts of rabbit heart, kidney, or skeletal muscle, and rabbit liver and spleen contain much less than do packed leucocytes.

Extracts of human and of guinea pig microphages show less bactericidal activity than rabbit cell preparations. Similar extracts of rat and mouse polymorphonuclear leucocytes contain no demonstrable phagocytin.

As indicated by its behavior on dialysis, on exposure to proteolytic enzymes, and on salt fractionation, phagocytin appears to be a protein with general properties characteristic of a globulin. It is clearly different from lysozyme and from properdin.

Although phagocytin is reasonably stable at temperatures of 65°C. and lower for several hours, solutions of it gradually lose bactericidal activity on standing for prolonged periods at 4°C. This instability, and also the ease with

which phagocytin is inactivated, presumably by adsorption, on exposure to a variety of materials, have thus far rendered fruitless efforts to isolate it.

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