CHARACTERIZATION OF LEUKIN: AN ANTIBACTERIAL FACTOR FROM LEUCOCYTES ACTIVE AGAINST GRAM-POSITIVE PATHOGENS*

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Antimicrobial substances have long been sought in normal tissues and fluids in attempts to elucidate mechanisms of natural immunity. Many investigators have extracted such materials from leucocytes during the last 65 years but little has been done to clarify the relationship of these substances to disease resistance. The lack of knowledge concerning the nature of the complex *in vivo* environment and the problems attendant to the isolation and purification of factors in tissue extracts have retarded progress.

Hankin (14), in 1891, first observed that leucocyte extracts exhibited an antibacterial action against the anthrax bacillus. Since this initial report, many other investigators have extracted antimicrobial products from leucocytes. Some of these reports dealt with heat-labile fractions which were similar to serum alexin and most active against Gram-negative bacteria (10, 11, 13, 14). However, the majority of the leucocyte extracts possessing antimicrobial activity are more closely allied with the beta lysins of serum (4, 10, 12, 21, 24, 25, 26, 35, 38, 47). These latter substances, termed "leukins" by Schneider (39), are relatively heat-stable, requiring temperatures of 65–80° for inactivation, and they are more active against Gram-positive bacteria. The chemical nature of these factors has not been defined other than to note that the antimicrobial activity of some resides in the globulin fraction of the leucocytes.

In much of the early literature dealing with extractions of these factors from leucocytes, the usual methods involved suspension of the cells in physiologic saline and then allowing the liberation of cellular components by freeze-thawing or heating to about 50° for one-half hour. More recently, acid extractions (4, 12) and sonic vibrations (8, 18) have been employed for this purpose.

It has been well established that neutrophils are attracted in large numbers to local sites of irritation and are seen to undergo rapid dissolution as the inflammatory reaction develops. The pH in such a local area gradually changes

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from alkaline to acid due to the accumulation of lactic acid which results from the altered metabolic activities of the leucocytes. This knowledge offered a rationale for the method employed here in the preparation of an antibacterial substance from neutrophilic leucocytes. It was thought that by allowing leucocytes to undergo *in vitro* autolysis in a simulated inflammatory environment, the possibility of obtaining a natural antibacterial substance rather than an artifact would be enhanced. By the use of such a method, a potent antibacterial factor was obtained and its chemical composition determined.

Materials and Methods

Preparation of the Leucocyte Factor.—

Normal rabbits received 400 to 750 ml. of physiologic saline by intraperitoneal injection. An automatic media-dispensing apparatus was used to pump the solution into immobilized rabbits. The peritoneal exudates of 20 to 25 rabbits were pooled for each extraction procedure. The leucocyte suspensions were collected 12 to 14 hours later by drainage through rubber tubing attached to a large bore needle. The peritoneal suspensions, consisting of 90 to 95 per cent neutrophils, were drained into 250 ml. plastic centrifuge bottles and immediately centrifuged at 200 gravity for 5 to 10 minutes. The volume of the pooled leucocyte suspensions was usually between 2500 and 3000 ml. The centrifugate was resuspended in physiologic saline and any fibrin clots were filtered off onto loose gauze. After one additional saline wash the leucocytes were resuspended in saline, distilled water, buffers or 0.02 to 0.5 per cent lactic acid in saline. Care was taken to maintain a temperature of 37° from the time of removal of the leucocytes from the rabbits until the extraction period was initiated.

Extraction of the leucocytes in the various fluids was carried out in Erlenmeyer flasks for periods of 12 to 96 hours at 37°. The pH of the extraction mixtures generally reached 2.5 to 3.0 within 24 hours in the unbuffered systems. In the buffered systems 0.5 to 1.0 M phosphate buffer at pH levels of 8.0, 7.0, and 6.0 were employed. Acetate buffer at 0.5 M concentration was employed at pH levels of 5.0 and 4.0. In some cases the extraction flasks were placed on a shaking apparatus during the incubation period to facilitate cellular disintegration. After extraction, cellular debris was centrifuged down at 1800 gravity for 45 minutes. The opalescent supernatant was brought to pH 7.0 with 2 N NaOH and the precipitate which formed was centrifuged at 1800 gravity for 30 minutes and discarded. The clear supernatant was dialyzed in the cold for 30 hours against three changes of distilled water. The resulting precipitate was centrifuged for 30 minutes at 1800 gravity and discarded. The supernatant solution was brought to pH 4.5 with N HCl and allowed to stand in the cold for 12 hours. The precipitate which developed was centrifuged at 1800 gravity for 30 minutes and discarded. The supernatant was lyophilized and weighed. The yield at this point was about 500 to 700 mg. of dry product. A solution of this material at a concentration of 0.2 per cent in distilled water was brought to pH 6.5 with N NaOH and half saturated with ammonium sulfate. After standing at room temperature for 4 hours the precipitate was centrifuged at 1800 gravity for 30 minutes and the supernatant discarded. The precipitate was resuspended in distilled water to the previous volume and dialyzed against distilled water until a negative Nessler test was obtained in the diffusate. The material in the cellophane bag was then lyophilized and weighed, the yield being 200 to 400 mg. This represented the final product which was tested biologically and studied chemically. An outline of the steps involved in this procedure is given in Fig. 1.

As a means for further purification a stepwise ammonium sulfate fractionation was performed on aliquots of preparations prior to the usual ammonium sulfate precipitation step.

An isoelectric point precipitation procedure (16) was employed in another attempt to improve the purity of the leucocyte factor.

Characterization of the Leucocyte Factor.—To test the antibacterial activity of the factor a stock pathogenic strain of *Micrococcus pyogenes* var. *aureus* was used. It was desired, in testing potency, to standardize activity on a weight basis. The unit activity of the factor was arbitrarily chosen as the least amount, in 10 ml. of growth medium, which prevented for 8 hours the outgrowth of an inoculum of 15 million cells from a 12 hour broth culture.

One and one-half strength nutrient broth (pH 8.2) was buffered with 1 ml. of 0.1 M phosphate at pH 8.2. The test systems and controls were incubated at 37° in screw-capped vials to which various amounts of the factor were added. Evidence of growth or growth inhibition was

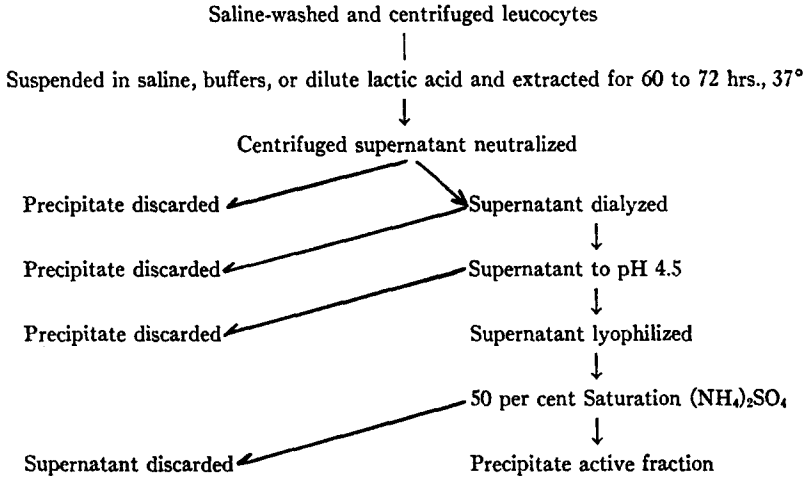

FIG. 1. General method for preparation of the leucocyte factor

determined by direct turbidity measurements in a Coleman Junior spectrophotometer at a wave length of 580 mμ.

In order to investigate the nature of the antibacterial property of the leucocyte factor a standard inoculum of 15 million cells of the stock strain of *M. aureus* was added to tubes containing 15 ml. of buffered nutrient broth. Twenty μg. of the factor were added to half the tubes, the other tubes served as growth controls. The tubes were incubated at 37° and triplicate plate counts made from 0.5 ml. aliquots of each tube at 0, 2, 4, 6, 10, and 16 hours.

Blockage of leucocyte factor antibacterial activity was examined when the acidic polymers desoxyribonucleic acid, ribonucleic acid, glutamyl polypeptide, and hyaluronic acid were added to the test systems just prior to inoculation with the stock strain. Turbidity readings were performed every 2 hours through a 10 hour period, during which time the ability of any particular acidic macromolecule to reverse growth inhibition could be observed. Normal growth and growth inhibition controls were included with each experiment.

The thermostability of the leucocyte product was examined in boiling distilled water at pH 5.0, 7.0, and 9.5. The optimum pH for antibacterial activity was determined using

phosphate buffer in the pH range from 6.0 to 8.5. The electrophoretic mobility of the material was measured in the portable apparatus of the American Instrument Co., Silver Spring, Maryland. The antibacterial selectivity of the product was tested against various Gram-positive and negative species. An enriched medium, Todd-Hewitt broth, was used when testing against the more fastidious microorganisms.

Crystalline trypsin at a concentration of 0.1 per cent was added to 2 ml. volumes of 0.1 per cent solutions of the leucocyte factor. The pH was adjusted to 7.8 and the mixtures incubated at room temperature for 12 hours or at 37° for 4 hours. The action of ribonuclease upon the biologic activity of the substance was also studied. The experimental conditions under which the enzyme acted have been described by Lancefield (23). The enzyme-treated extracts were then examined for alteration of antibacterial potency. The effect of $MgSO_4$ upon the antibacterial power of the leucocyte factor was also investigated, using salt concentrations of 50 and 100 μg. per ml. Ultraviolet absorption patterns were determined with the Beckman spectrophotometer, model DU, employing 0.05 per cent solutions of the factor.

An amino acid analysis was performed following the method of Stein and Moore (31, 32). The dowex 50-X4 resin (-400 mesh) was used in the column. The leucocyte product was weighed from the lyophilized state in an amount of 6.25 mg. The product was hydrolyzed in a vacuum-sealed glass tube with 3.5 ml. of constant boiling hydrochloric acid for 48 hours at 110°. The hydrolysate was washed carefully from the tube into a round bottom flask and vacuum distilled with three additions of distilled water. After the third distillation the residue was brought down to approximately a 1 ml. volume and carefully washed into a 25 ml. volumetric flask with citrate buffer, pH 3.1, and brought to volume with buffer and distilled water. The final pH of the diluted sample was 2.2. The sample was then filtered through a fiber glass filter pad and stored in the referigrator until used.

Identification of the various amino acid peaks was accomplished by comparison with previously obtained results with known amino acids whose relative positions had been discerned by paper chromatographic methods (3). The total nucleic acid content of the leucocyte factor was determined from the phosphorus value (9), after three extractions in equal parts of ether and alcohol. Desoxyribonucleic acid was measured by a modification of the Dische test (40). The ribonucleic acid value was arrived at by subtraction of the Dische test result from the total nucleic acid value. Total carbohydrate was measured by the anthrone test (33) after deproteinization with trichloroacetic acid, and was based on glucose equivalents. The total protein content was determined from the amino acid assay and by the micro-Kjeldahl method (20).

EXPERIMENTAL RESULTS

Preparation of the Leucocyte Factor.—Extraction of leucocytes in the various solutions yielded antibacterial fractions differing both in quantity and quality. When leucocytes were extracted in solutions buffered at pH 8.0, 7.0, and 6.0 for periods of 12 to 24 hours, small amounts of two different antibacterial factors were obtained. One factor rapidly lost its antibacterial power upon standing in solution at 3°. The pH for greatest activity of this factor was 6.0 to 6.5. The other antibacterial factor which was stable and obtained in better yields in unbuffered or dilute lactic acid solutions was the one chosen to be investigated.

Although it could be obtained from neutrophils by extraction in any of the different solutions during various time intervals, best yields resulted when complete cellular disintegration was accomplished. Greatest yields were ob-

tained when the leucocytes were extracted at 37° in dilute lactic acid (0.02 to 0.5 per cent) in saline for 60 to 72 hours on a shaking apparatus.

Attempts to concentrate the activity further by three different means proved unsatisfactory. The step-wise ammonium sulfate procedure yielded three fractions which, when tested biologically, were less active than the whole half-saturated product. By recombining the three fractions the potency was returned to that of the best preparations. Thus, the separation of the 50 per cent ammonium sulfate precipitate into three fractions had the effect of diluting rather than concentrating antibacterial power. The isoelectric point precipitation procedure gave three to four fractions which contained some activity

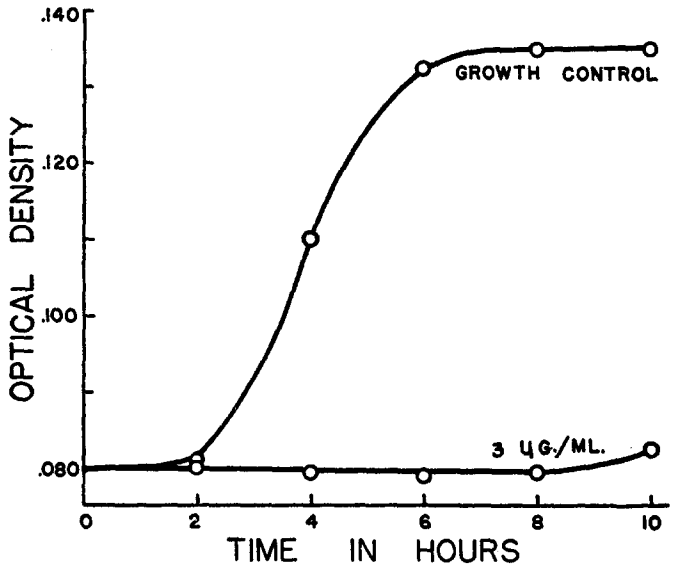

FIG. 2. The unit activity of one preparation of the leucocyte factor.

but none of which appeared to concentrate the property. Electrophoretic runs were performed in an attempt to separate fast-moving boundaries. However, because of the limited water solubility of the leucocyte factor the patterns were difficult to follow. Several small, rapidly diffusing boundaries were seen to migrate toward the negative pole at pH 7.0 but not in sufficient quantity to warrant recovery efforts.

Characterization of the Leucocyte Factor.—Fig. 2 illustrates the unit activity of one preparation of the material. In this case, 3 µg. per ml. prevented for 8 hours the outgrowth of the stock strain of *M. aureus*. The unit activities of these various leucocyte preparations varied between 2 and 5 µg. per ml.

In testing the nature of the antibacterial property, it was found that the

factor exhibited a bactericidal effect upon *M. aureus*. Table I contains the results of this finding. Within 2 to 4 hours over 99 per cent of the initial population was killed. The eventual outgrowth which always occurred in the presence of low concentrations may have been the result of the existence of resistant forms in the population. The possibility also exists that the effective concentration of leucocyte factor was reduced as a result of binding to dead cells. If bacteria were exposed to relatively high concentrations of the factor, (25 to 50 μg. per ml.), the cultures failed entirely to grow out.

The material was found to be very heat-stable at acid or neutral pH but unstable at the alkaline reaction. Boiling a 0.1 to 0.2 per cent solution of the factor for 90 minutes at pH 5.0 or 7.0 did not reduce its bactericidal power and only a slight reduction in potency resulted after 2 hours of boiling. Solutions remained clear during the heating periods. Solutions adjusted to pH 9.5 became cloudy and precipitated out soon after boiling was begun. No antibac-

TABLE I

The Nature of the Antibacterial Property of the Leucocyte Factor

Time	Growth controls	Leucocyte factor
hrs.	*Av. No. bacteria*	*Av. No. bacteria*
0	1.5×10^6	1.5×10^6
2	9.5×10^6	1.1×10^5
4	5.3×10^7	1.0×10^4
6	TNC	3.6×10^4
10	TNC	1.1×10^6
16	TNC	TNC

terial activity could be detected in solutions heated for 15 to 30 minutes at boiling temperature even when high concentrations of up to 100 μg. per ml. were tested.

The acidic polymers glutamyl polypeptide, hyaluronic acid, and desoxyribonucleic acid effectively reversed the bactericidal action of the leucocyte product. Ribonucleic acid was much less effective and only large ratios of acid to leucocyte factor caused partial interference. Fig. 3 shows the blockage of antibacterial action by glutamyl polypeptide when the latter was added in a ratio 10:1.

The optimum pH for greatest antibacterial action was found to be 8.2 as seen in Fig. 4. At pH levels of 8.5 and above, the growth controls did not grow out normally. Although the pH optimum was 8.2, the factor was only slightly less active at pH values of 7.2 and 6.5.

The product was tested against several species of bacteria with the results listed in Table II. All Gram-positive species were inhibited, while none of the Gram-negative species were affected adversely at the concentration of 30

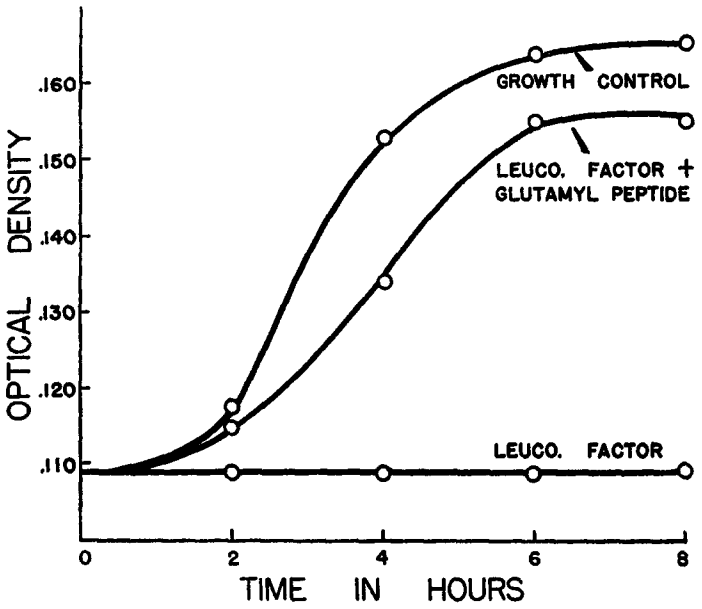

FIG. 3. The interference by glutamyl polypeptide with the bactericidal property of the leucocyte factor against the staphylococcus. The ratio of acid to factor is 10:1.

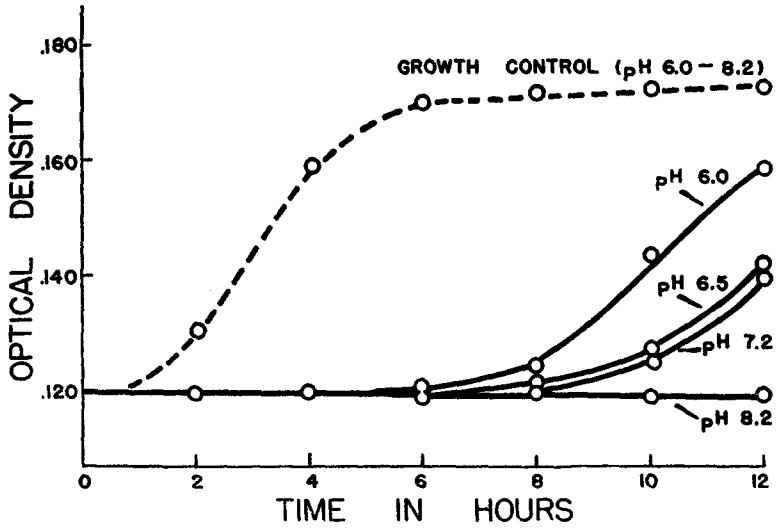

FIG. 4. The optimum pH for bactericidal action of the leucocyte factor.

μg. per ml. Only concentrations higher than 100 μg. per ml. proved inimical to the Gram-negative bacteria tested.

The complex growth medium required by certain of the Gram-positive bacteria reduced somewhat the effectiveness of the leucocyte factor. Whereas

M. aureus could be effectively inhibited by 2 to 3 μg. per ml. in nutrient broth, inhibition occurred only at concentrations of approximately 10 μg. per ml. in Todd-Hewitt broth. It is interesting to note that the type II pneumococcus, either in the rough or smooth phase was very susceptible to the leucocyte product. Microscopic examination of the exposed pneumococcal cells showed that the large majority were lysed within 4 hours.

Exposure of the leucocyte factor to trypsin for a period of 12 hours at room temperature or for 4 hours at 37° abolished the bactericidal activity. The enzyme ribonuclease did not alter antibacterial action. Neither enzyme exhibited an antibacterial property of its own at the concentrations utilized.

TABLE II

*Range of Antibacterial Selectivity of the Leucocyte Factor**

Bacterial species	Growth effect
Micrococcus aureus	Inhibition
Type II pneumococcus	"
Type 28 streptococcus	"
Type 4 streptococcus	"
Streptococcus faecalis	"
Type 18 streptococcus	Partial inhibition
Salmonella typhosa	No inhibition
Escherichia coli	" "
Vibrio comma	" "
Proteus vulgaris	" "
Shigella paradysenteriae	" "

* 30 μg. per ml.

$MgSO_4$ did not interfere with bactericidal action at concentrations which had previously been shown to reverse the antibacterial properties of two thymus peptides (1, 17). Two ultraviolet absorption peaks were demonstrated. Fig. 5 shows two curves, the lower curve represents the untreated leucocyte product. The peak at 258 mμ reflects the presence of nucleic acid while the peak at 275 mμ represents protein material. The upper curve was obtained after the factor had been treated with ribonuclease. It can be observed that the protein peak has been shifted to the right to 278 mμ suggesting a protein-nucleic acid association.

Table III summarizes the characteristics of the leucocyte factor which have been studied. Prolonged dialysis led to a gradual loss in activity presumably due to loss through the dialyzing membrane. When a solution of the factor at a concentration of 0.1 per cent was passed through Selas filter No. 0.02 no bactericidal activity was found in the filtrate probably because of adsorption to the filter. The factor had a limited water solubility, the maxi-

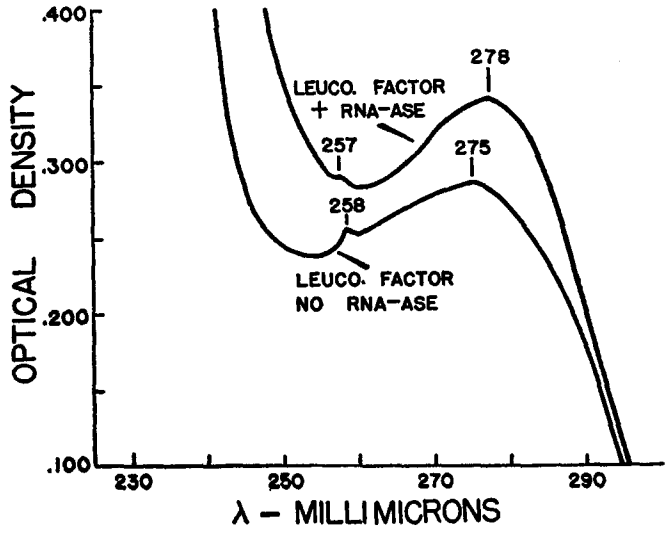

FIG. 5. Ultraviolet absorption patterns of the leucocyte factor (0.05 per cent) before and after treatment with ribonuclease.

TABLE III

Characterization of the Leucocyte Factor

Property	Effect
1 Dialyzability	1. Slowly dialyzable
2. Filterability	2. Not passed by Selas filter
3. Heat stability	3. Slight inactivation after boiling 2 hrs. at acid or neutral pH. Inactivated in 30 min. at pH 9.5
4. Solubility	4. Soluble in water to 0.4 per cent.
5. Inhibition of activity by acidic polymers	5. DNA and glutamyl peptide block action, hyaluronate to a lesser degree
6. Optimum pH of action	6. pH 8.0 to 8.2
7. Electrophoretic mobility of one boundary	7. 15.7×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ toward the negative pole, pH 7.0
8. Antibacterial potency	8. 2–10 μg./ml., depending upon the nutrient medium
9. Antibacterial selectivity	9. Active against gram positive pathogens: Group A streptococci, staphylococci, pneumococcus II. Not active against Gram-negatives: *E. coli*, *V. comma*, *S. typhosa*, *P. vulgaris*, *Sh. paradysenteriae*
10. Action of enzymes	10. Trypsin destroys activity; ribonuclease does not
11. Action of $MgSO_4$	11. $MgSO_4$ does not interfere with bactericidal action at a concentration of 100 μg./ml.
12. UV absorption	12. Absorption peaks at 258 and 277 millimicrons at 0.05 per cent concentration

mum concentration being 0.3 to 0.4 per cent at neutral to acid pH. The electrophoretic mobility of one fast moving boundary was found to 15.7×10^{-5} $cm.^2$ $volt^{-1}sec.^{-1}$ Cacodylate buffer was used at pH 7.0 and at an ionic strength of 0.08. The current applied was 10 milliamperes.

Many of the characteristics listed in Table III suggest that the antibacterial factor might be a basic protein or polypeptide. Although the preparation obtained was not homogeneous, an amino acid analysis was performed to

TABLE IV

Amino Acid Analysis of the Leucocyte Factor

1. Column chromatographic analysis

Amino acid	Amino acid on total weight basis	Amino acid per 100 gm. total protein
	per cent	*gm.*
Aspartic acid	5.40	6.2
Threonine	3.86	4.4
Serine	3.90	4.5
Proline	5.61	6.4
Glutamic Acid	7.83	9.0
Glycine	5.42	6.2
Alanine	4.19	4.8
Valine	8.60	9.9
Methionine	0.85	1.0
Isoleucine	4.03	4.6
Leucine	7.04	8.1
Tyrosine	1.86	2.1
Phenylalanine	4.36	5.0
Lysine	3.91	4.5
Histidine	3.57	4.1
Arginine	14.63	16.8
Ammonia	2.13	2.5
Total	87.19	100.1

discover whether it contained disproportionate amounts of the basic amino acids. Table IV presents the result of this assay, in which it is seen that the basic amino acid arginine predominated, representing nearly 17 per cent of the total protein. The other two basic amino acids, lysine and histidine, were not present in unusual quantities.

Total Composition of the Leucocyte Factor.—The total protein value for the product as determined by the amino acid analysis was approximately 87.2 per cent. An average protein value determined by the micro-Kjeldahl method was found to be 90.0 per cent. The presence of nucleic acids may have accounted for the slightly higher value obtained by the latter method.

Total nucleic acids determined from the phosphorus content 0.90 per cent represent 8.2 per cent of the leucocyte factor. From the Dische test, desoxyribonucleic acid appeared to be present in a concentration of 1.5 per cent. The ribonucleic acid content as calculated by subtraction of the desoxyribonucleic acid value from the total nucleic acids represented 6.7 per cent of the total weight.

Total carbohydrate was determined by the anthrone test and found to be 4.0 per cent. The total constituents of the factor are enumerated in Table V. More than 99 per cent of the product was accounted for on a weight basis.

TABLE V

Total Constituents of the Leucocyte Factor

1. Total protein:	
(*a*) By amino acid analysis	87.2 per cent
(*b*) By micro-Kjeldahl method	90.0 per cent
2. Total nucleic acids: (from total P—8.2 per cent)	
(*a*) DNA by Dische method	1.5 per cent
(*b*) RNA difference	6.7 per cent
3. Total carbohydrate:	
(*a*) Glucose equivalents by Anthrone test	4.0 per cent
4. Total components of sample:	
(*a*) Protein—	87.2 per cent
(*b*) Nucleic acids—	8.2 per cent
(*c*) Carbohydrate—	4.0 per cent
	99.4 per cent total recovery

DISCUSSION

Two different antibacterial factors were obtained in preliminary experiments when leucocytes were permitted to break down at 37° in buffered physiologic saline during a 24 hour extraction period. One such substance was quite unstable and most effective at acid reaction. Examination of the cellular debris at this time showed that no intact cells were present although most cell nuclei were whole, indicating that this factor may have been present in the cytoplasm. This material was probably related to some antibacterial substances reported in the early literature (10, 11, 13, 44) and to phagocytin (18, 19).

The other antibacterial substance studied here was very stable and most active at pH 8.2. If leucocytes were extracted without buffer, the pH was found to reach 3.0 or 2.5 within 24 hours, probably owing to the accumulation

of lactic acid. Under these circumstances greater yields of the stable substance were obtained, particularly when the neutrophils were extracted for 2 to 3 days. Because of the acid reaction which develops in inflammatory sites and because of the better yield of leucocyte factor in acid extracts, leucocytes were subsequently suspended in dilute lactic acid–physiologic saline solutions and extracted for 60 to 72 hours at 37°. No intact cell nuclei could be detected after this prolonged extraction, indicating that the antibacterial factor originated in the nuclear fraction.

The lower the extraction pH, the greater was the yield of antibacterial factor obtained. This may have been the result of a greater release of protein from nucleic acids in the acid environment. If cellular debris which had been extracted for 2 to 3 days at near neutral pH was then extracted in lactic acid at pH 2.5 to 3.0, more of the antibacterial factor was obtained in the supernatant. On the other hand, the cellular debris of leucocytes which had been extracted in lactic acid for 3 days did not give up any more antibacterial material upon further acid extraction.

Unfortunately the methods used to further purify the final leucocyte product failed to concentrate antibacterial activity. However, the preparation proved quite potent in low concentrations suggesting that it probably contained a significant amount of the desired product. The fact that the activity was decreased rather than increased by fractionation procedures indicated that the substance may have been bound to impurities or composed of different sized molecules some of which were more active than others.

Many of the characteristics of the leucocyte factor suggested that it was a basic protein or polypeptide. In several of these characteristics is resembled lysozyme and the basic thymus peptide of Bloom *et al.* (4). However, it differed from it in certain important respects. It was low in lysine and high in arginine which was opposite to the finding with the thymus peptide. Its antibacterial selectivity differed from the thymus peptide in that the pneumococcus was not susceptible to the peptide while two Gram-negative species were susceptible (42). $MgSO_4$ interfered with thymus peptide activity (1) but not with the leucocyte factor, and the ultraviolet absorption peaks for the two substances were different (4). The electrophoretic mobility of the factor appeared to be greater than that of the tissue peptide (4). Although lysozyme activity was found in crude extracts of the leucocytes, none remained in the final product because of the 50 to 60 hour dialyzing period which was employed.

Many characteristics of the leucocyte factor are shared by protamines. The protamines are known to contain large amounts of arginine and they are acid and heat-stable (22). They are inhibited by large acidic polymers (45), more active against Gram-positive bacteria (30, 34), most active at pH 8.1 (30) and inactivated by trypsin (22). It is on these bases that the antibacterial factor was tentatively identified as a protamine or protamine derivative. That nuclei of polymorphonuclear leucocytes contained a protamine was

first shown by Miescher in 1869. He extracted a nuclein from human pus cells and separated the protein portion which he characterized and later named protamine (29).

The method of leucocyte extraction which usually involved an incubation period of 3 days in a lactic acid environment at pH 2.5 to 3.0 may have been conducive to the formation of protamine split products, possibly because of the acid environment, or by the action of tissue cathepsins (43) or acid proteases such as the one found in lymphatic tissue by Hess *et al.* (15). Furthermore, the inhomogeneity of the electrophoretic patterns may have been partly the result of migration of different sized protamine derivatives. Finally, the *in vitro* antimicrobial power of the leucocyte factor was much greater than that reported for intact protamine molecules (30, 27, 45). The splitting of the protamine molecule might expose more of the basic ammonium groups, accounting for increased activity. A situation analogous to this is suggested by the work of Bloom and coworkers with the basic thymus peptide believed to have originated from a histone (4, 45).

The review article by Bartholomew and Mittwer (2) summarized the literature which established that the cell surfaces of Gram-positive bacteria contain ribonucleic acid. This feature could provide these forms with a greater negatively charged surface than is the case with the Gram-negative bacteria. Thus, the positively charged ammonium groups on the leucocyte factor may be afforded a greater opportunity for electrostatic attraction to the cell surface with a consequent disruption of cell metabolism. This may account for the selectivity of action of the leucocyte product upon the Gram-positive species.

The finding that acidic polymers could block the antibacterial property is of interest. The significance of this result has been discussed in a previous publication (41) in which it was postulated that acidic bacterial capsules may offer protection to invading microorganisms by reducing the effectiveness of basic antibacterial factors. However, in most instances the natural defenses of the host enable it to overcome invading microorganisms. One facet of this natural defense system may involve the elaboration of sufficient amounts of basic tissue substances to offset this advantage of the parasite.

The question of whether phagocytes, particularly neutrophils, offer resistence to infectious agents by virtue of their phagocytic activity alone is unsettled. Metchnikoff believed that the primary function of phagocytes in defense was the act of phagocytosis (28). Contemporary qualified support for this concept is afforded by Wood (46).

In view of the numerous reports concerning the antimicrobial action of the variously extracted neutrophilic substances, an additional defense role for these cells has been suggested. Ribbert (36), Dubos (7), and Cromartie *et al.* (5, 6), among others, have observed what appeared to be an extracellular or preparatory action of neutrophils upon invading microorganisms. Shortly

after microbial agents were introduced into animal tissues they were seen to be surrounded, but not immediately phagocytized, by numerous polymorphonuclear leucocytes in various stages of dissolution. It is possible that the degenerating leucocytes in acidic inflammatory sites elaborate substances inimical to the invading organisms thus rendering them more susceptible to ultimate destruction and phagocytosis. It should be emphasized that this effect would most probably be localized; *i.e.*, restricted to the immediate environment of the leucocytes and microorganisms. The possibility that protamine derivatives might arise within injured neutrophils is made more tenable by the findings of Rous (37) that certain areas within polymorphonuclear leucocytes exhibited a pH as low as 3.0. Thus, the opportunity for the elaboration of a leucocyte factor such as described above seems reasonable.

The leucocyte product which has been investigated in this study is very likely similar to the relatively heat-stable substances which have been reported in the early literature. The greater heat stability of the substance reported here may be a consequence of the greater degree of purity obtained. Early work on the heat stability of many of the leucocyte factors usually involved heating periods in whole or diluted serum. It is probable that the product described here would be less heat-stable if heated in the presence of serum. Such a basic substance would be bound rapidly by serum proteins and its activity decreased. That this would be the case is indicated by the finding that a complex medium like Todd-Hewitt broth reduces the potency of the factor somewhat.

Since the relatively heat-stable leucocyte factors reported in the early literature were most active against Gram-positive bacteria and had been named "leukins" by Schneider (39) it seems superfluous to suggest a new term for the related leucocyte factor studied here.

Several investigators have described less heat-stable leucocyte substances which were most active against Gram-negative bacteria. The most recent report is that of Hirsch (18, 19), who named the factor "phagocytin." This substance was shown to be bactericidal for many Gram-negative species but not for Gram-positive pathogens. Phagocytin was shown to be most active at acidic reactions but its chemical nature has not yet been ascertained.

The elaboration by polymorphonuclear leucocytes of two different antibacterial factors may assume a significant role in the elucidation of a natural defense mechanism against both Gram-positive and Gram-negative bacteria. The leukin(s) may act against Gram-positive species while the phagocytin(s) may attack Gram-negative forms.

SUMMARY

A method has been described for the preparation of a potent antibacterial factor from rabbit polymorphonuclear leucocytes. Upon characterization, the

factor was found to possess many properties in common with basic proteins. The amino acid analysis revealed that it contained a relatively large amount of arginine (17 per cent) and small amounts of the other two basic amino acids. It has therefore been identified as a protamine or protamine derivative.

The leucocyte factor was very active against all Gram-positive pathogens tested but exhibited little or no action against Gram-negative species. A possible explanation of this phenomenon has been discussed. The factor was very heat-stable at acid and neutral pH and its staphylococcidal activity was blocked by glutamyl polypeptide, hyaluronic acid, and desoxyribonucleic acid.

Because of the apparent similarity of the product studied here to other poorly defined leucocyte factors which had been termed leukins in the early literature, it is suggested that the name leukin be retained for it. The possible significance of this leukin in natural immunity has been discussed.

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