DEGRANULATION OF POLYMORPHONUCLEAR LEUCOCYTES FOLLOWING PHAGOCYTOSIS OF MICROORGANISMS*

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Polymorphonuclear leucocytes contain at least three antimocrobial agents: phagocytin, lysozyme, and acid (1). Results presented in the preceding communication (2) demonstrate that the major portion of rabbit leucocytic phagocytin is located in granules isolated from the cytoplasm of these cells. These granules can be lysed and the phagocytin effectively extracted from them by exposure to dilute acid.

Previous studies (3, 4) have established that the pH surrounding microorganisms within leucocytes is near 5, a level sufficiently acid to liberate phagocytin from granules. This fact, considered along with the well known increased glycolytic rate and increased lactic acid production accompanying active phagocytosis (5), led us to examine the possibility that cytoplasmic granules in polymorphonuclear white cells might be lysed following engulfment of microorganisms or other particles.

Methods

Leucocytes were obtained from peritoneal exudates in rabbits and from human blood by techniques described previously (6).

Bacillus subtilis and *Salmonella typhimurium* RIA were cultured on penassay agar slants. The streptococcus employed, strain C748 (Group A, Type 4, hyaluronidase$^+$, capsule$^-$, M$^-$), was grown overnight in Todd-Hewitt broth containing 0.3 per cent bovine albumin. *Mycobacterium smegmatis* was cultured for 48 hours in penassay broth with frequent shaking and large clumps were removed by sedimentation. For use in the phagocytic tests, all cultures were washed by centrifugation in Gey's balanced salt solution, and finally suspended in Gey's solution containing 10 or 20 per cent normal rabbit serum.

Zymosan (Fleishmann Laboratories, Standard Brands, Inc., New York, Lot 4B145) was also washed in Gey's solution and suspended in serum-Gey's solution as described for the bacteria above.

Initially washed bacteria were mixed with polymorphonuclear leucocytes in suspension and stained smears were examined to determine whether engulfment of microbes was associated with a change in the cytoplasmic granules. The observations did indeed suggest that phagocytosis of large numbers of bacteria was followed by degranulation; however, under

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these conditions severe clumping of the white cells always occurred, and this aggregation made it difficult to demonstrate the phenomenon convincingly. Also, degranulation of leucocytes in this system might have been related primarily to clumping of the cells rather than to phagocytosis. Efforts to prevent or reverse leucocyte aggregation by use of proteases, chelating agents, or a plasma clot technique were not successful.

The procedure finally developed for observation of this phenomenon involved sedimentation of leucocytes onto a glass surface, and, after washing, sedimentation of microbes or particles onto the white cells *in situ*. Felsen quadrant Petri dishes were adapted to serve as moist chambers by fitting a soft rubber gasket about the upper lip of the bottom dish. Water was placed in the bottom of each quadrant and a clean glass slide was placed on the ridges. After incubation at 38°C. for 30 minutes or longer to permit equilibration and saturation of the air with water, 0.2 to 0.3 ml. of white cell suspension was placed in the center of the slide and allowed to spread naturally. Leucocytes in heparinized human or rabbit blood did not regularly adhere to the slide, whereas satisfactory preparations could be made from rabbit exudate cells or washed leucocytes isolated from human blood. Rabbit peritoneal exudates containing approximately 0.1 mg./ml. heparin were used within 1 hour of their collection; washing of exudate cells was not necessary. Human white cells collected from blood by a dextran sedimentation procedure (6) were washed once with saline in the centrifuge and suspended in Gey's solution—10 per cent human serum for sedimentation onto the glass slides. After incubation at 38°C. for 30 minutes, the slides were agitated gently in Gey's solution; leucocytes had settled to form a firmly adherent patch of cells. Suspensions of white cells at approximately 5000 per c.mm. gave suitable cell densities on the slides. After washing the preparations were immediately overlaid with 10 to 20 per cent rabbit or human serum in Gey's solution or with the same medium containing bacteria or inanimate particles. Care was taken not to permit the cell sheet to dry out during washing and addition of the test medium. The slide preparations were then again incubated at 38°C. for 30 minutes, or in some instances as noted in the tables, for longer periods of time. This procedure permitted ready phagocytosis of the bacteria employed, and eliminated the problem of leucocyte clumping.

Finally the slides were removed and rinsed in Gey's solution containing 10 to 20 per cent serum. If allowed to drain and dry on standing, the preparations were unsatisfactory for microscopic observation, since the leucocytes retracted into small dense spherical bodies. A technique was developed for rapid fixation of the adherent cells in a well spread state. This was accomplished by flash drying with an air jet. Compressed air was filtered through cotton and led into a 3 inch long blunt-end 18 gauge needle to secure a forceful jet of air. This air jet was then passed over each slide immediately after its final washing; drying was accomplished almost instantaneously. Care was required that the air jet not be so forceful as to blow off or deform the cell sheet. The photomicrographs (Figs. 1 A to 1 E) demonstrate the excellent preservation of cell structure in a form suitable for microscopic observation obtained by this technique.

Staining was performed with Wright's stain, with modifications which gave the best definition of cytoplasmic granules and of engulfed particles. Each slide was flooded with 0.8 to 1.0 ml. of Wright's stain. After 1 minute, 0.4 to 0.5 ml. of distilled water (pH approximately 5.0) was added and mixed well by rocking the slide. Staining was continued for 5 minutes after addition of water.

Accurate counting of cytoplasmic granules in rabbit polymorphonuclear cells was often possible. Usually cells were divided into three categories: those with more than 30 granules (fully granulated), those with 10 to 30 granules (partially degranulated), and those with less than 10 granules (markedly degranulated). Granules in human neutrophiles were too small to permit accurate counting, but it was nonetheless possible to divide these cells into three categories of markedly degranulated, partially degranulated, and fully granulated.

RESULTS

Degranulation of Rabbit Polymorphonuclear Leucocytes Following Phagocytosis of Various Microorganisms

Table I presents results of an experiment in which rabbit polymorphonuclear leucocytes adherent to a glass slide were allowed to engulf chains of Group A streptococci (Type 4). Control specimens not exposed to streptococci revealed no change in granulation throughout the 90 minute period of observation, whereas the leucocyte preparations engulfing streptococci showed striking and progressive loss of cytoplasmic granules (illustrated in Figs. 1 A and 1 C). In this experiment, a large streptococcal inoculum was added, so that approximately 90 per cent of the white cells contained microbes at the 30

TABLE I

Influence of Duration of Incubation on the Degranulation of Rabbit Polymorphonuclear Leucocytes Following Phagocytosis of Bacteria

Duration of incubation	Microorganism presented for phagocytosis	Percentage of polymorphonuclear leucocytes containing the following No. of granules per cell		
		Less than 10	10–30	More than 30
min.				
30	None	0	9	91
	Type 4 streptococcus	23	60	17
60	None	0	9	91
	Type 4 streptococcus	57	41	2
90	None	0	8	92
	Type 4 streptococcus	76	23	1

minute sampling. Essentially all granulocytes in the 60 and 90 minute specimens had engulfed microorganisms, and in addition the number of streptococcal chains per leucocyte increased with passage of time. There was thus evidence for continuing phagocytosis in the system, and it was impossible to determine whether the progressive degranulation observed was related to the duration of incubation following ingestion of particulate matter, or to the quantity of material engulfed, or to both.

Similar experiments performed employing *Mycobacerium smegmatis*, *Bacillus subtilis* and *Salmonella typhimurium* in various concentrations are summarized in Table II. A single sample of each specimen was examined after incubation for 45 minutes. The various bacteria were approximately equally potent as degranulating agents, and the degree of degranulation in each instance was closely correlated with the degree of phagocytosis. Marked degranulation was seen whenever 50 per cent or more of the white cells had ingested microbes. There

was also a good correlation between the average number of bacteria per infected cell and the degree of degranulation.

Wright's stains of those specimens in which the polymorphonuclear leucocytes had engulfed large numbers of bacteria revealed changes other than degranulation in some of the phagocytes: shrinking and rounding of the entire cell, vacuolization of cytoplasm, and deformity and altered staining of the nucleus. Examination of such specimens by supravital methods established that the cells remained alive for more than 1 hour. Nuclear staining with trypan blue did not occur. In warm stage preparations studied by phase contrast micros-

TABLE II

Influence of Size of Inoculum of Various Microorganisms on the Degranulation of Rabbit Polymorphonuclear Leucocytes

Microorganism	Approximate No. of bacteria applied	Approximate percentage of PMN containing bacteria	Approximate average No. of intracellular bacteria per infected cell	Percentage of polymorphonuclear leucocytes containing the following numbers of granules per cell		
				Less than 10	10–30	More than 30
Mycobacterium smegmatis	5×10^6	50	3	14	47	39
" "	5×10^5	10	1	0	19	81
Bacillus subtilis	5×10^7	>90	10	74	25	1
" "	5×10^6	60	2	7	53	40
" "	5×10^5	10	1	0	18	82
Salmonella typhimurium RIA	10^8	>90	>10	81	19	0
" " "	10^7	80	5	20	78	2
" " "	10^6	10	1	1	14	85
None	—	—	—	0	9	91

copy, the heavily infected leucocytes differed from normal ones only in that they tended to remain somewhat rounded and migrated poorly or not at all. Cytoplasmic granules were not visualized clearly under phase contrast, so that observations could not be made on degranulation following phagocytosis in living white cells under constant observation.

Studies on Degranulation of Rabbit Polymorphonuclear Leucocytes Following Ingestion of Inanimate Particulate Matter and Following Exposure to Endotoxin

Repeated efforts were made to observe rabbit polymorphonuclear leucocytes for degranulation following ingestion of small quartz particles (1 to 3 μ) and of charcoal preparations. Phagocytosis of these materials was poor or absent in several test systems and no conclusions could be drawn.

Zymosan, a preparation of yeast cell wall, was readily engulfed by leucocytes in the standard slide sedimentation technique, and phagocytosis of zymosan particles by rabbit polymorphonuclear leucocytes was followed by loss of cytoplasmic granules, as illustrated in Fig. 1 D.

Investigation of a possible degranulating action of bacterial endotoxin on leucocytes was undertaken because of the many similarities in effects on leucocytic functions (7) of these lipopolysaccharides and of intact microorganisms.

The endotoxin employed, a highly purified material kindly supplied by Dr. O. Westphal, had been used for previous observations on leucocytic function and metabolism (7). It was suspended well in Gey's solution at 1 mg./ml. and allowed to stand in the cold with frequent mixing for 2 hours. The small amount of insoluble residue was then removed by centrifugation at 25,000 *g* for 15 minutes. The water-clear supernatant was added to serum-Gey's solution and overlaid on rabbit polymorphonuclear leucocytes sedimented on a glass slide. Microscopic observations were made after various periods of incubation at 38°C.

Final concentrations of endotoxin as high as 200 μg. per ml. failed to cause detectable change in general morphology or in granule content of rabbit polymorphonuclear leucocytes during periods of incubation up to 3 hours.

Relationship between Quantity of Material Engulfed by Rabbit Polymorphonuclear Leucocytes and the Degree of Degranulation Which Follows

Employing the slide sedimentation technique, additional experiments were performed to study quantitative aspects of leucocytic degranulation following phagocytosis of streptococci or zymosan.

The inoculum was adjusted so that approximately half of the white cell population engulfed particles. On each specimen degranulation was estimated by examining at least 100 cells which had ingested material and a like number of phagocytes which had not. In addition, polymorphonuclear leucocytes containing zymosan were divided into two groups: those containing only one zymosan particle and those containing 2 to 5 particles. Zymosan was especially suitable for this type of quantitative examination, since the particles were readily seen within phagocytes, and since rapid intracellular digestion of the zymosan did not occur. The object of analysis by this method was to eliminate the possibility that degranulation of leucocytes was due to microbial products liberated into the medium, rather than due directly to engulfment of particles.

As is shown in Table III, polymorphonuclear leucocytes which had ingested streptococci showed marked degranulation, whereas cells from the same population on the same slide which had not engulfed bacteria had a distribution of granules in their cytoplasm essentially the same as that of a control preparation with no added microorganisms. Non-phagocyting white cells in the system containing zymosan also remained normally granulated. Those leucocytes containing only 1 zymosan particle were for the most part partially degranulated, whereas those engulfing 2 or more zymosan bodies showed marked loss of cyto-

plasmic granules, thus establishing a relationship between quantity or numbers of particles ingested and the degree of degranulation.

Degranulation of Human Neutrophilic Leucocytes Following Phagocytosis of Streptococci or Zymosan

In contrast to the large distinct granules found in the cytoplasm of the rabbit polymorphonuclear leucocyte, the corresponding human cells presented a cytoplasm teeming with tiny neutrophilic or slightly acidophilic granules in slide sedimentation preparations stained by Wright's. It was not possible to count granules in these human cells, but arbitrary division of them into three

TABLE III

Demonstration of a Relationship between Degranulation of Rabbit Polymorphonuclear Leucocytes and Quantity of Material Engulfed by Them

Material presented for phagocytosis	Polymorphonuclear leucocytes examined	Percentage of cells with the following No. of granules per cell		
		Less than 10	10–30	More than 30
None	All	0	8	92
Type 4 streptococci	Those containing streptococci	40	48	12
" " "	Those containing no streptococci	0	7	93
Zymosan	Those containing 2–5 zymosan particles	51	42	7
"	Those containing 1 zymosan particle	3	52	45
"	Those containing no zymosan	0	10	90

groups (heavily, moderately, and lightly granulated) permitted assessment of the relationship between phagocytosis and granule content. Results of an experiment directed towards this point, presented in Table IV, were quite similar to those obtained for rabbit cells, and demonstrated clearly reduction in granule content of human polymorphonuclear leucocytes following ingestion of streptocci or of zymosan particles.

Morphologic Features of Leucocyte Degranulation Following Phagocytosis

Figs. 1 A and 1 B show the appearance of normal rabbit polymorphonuclear leucocytes in slide sedimentation preparations stained by Wright's. Included among the granulocytes in illustration B is a rabbit eosinophile; the eosinophile was a larger cell with more basophilic cytoplasm, and cytoplasmic granules larger in diameter and of a slightly different hue than those of the polymorphonuclear phagocytes.

Illustrations C and D demonstrate degranulation of rabbit polymorphonuclear leucocytes following ingestion of streptococci and of zymosan. Zymosan particles appeared as faintly blue bodies surrounded by a large clear zone. This clear zone was probably cell wall material of the zymosan rather than a cytoplasmic vacuole, since it had the same appearance in extracellular zymosan examined by phase contrast microscopy.

Illustration E shows human polymorphonuclear leucocytes, some of which had engulfed zymosan. Granules in the cytoplasm of these cells were much smaller and less distinct than those of the corresponding rabbit cells, but close

TABLE IV

Reduction in Numbers of Granules within Human Polymorphonuclear Leucocytes Following Phagocytosis of Streptococci or Zymosan Particles

Material presented for phagocytosis	Human neutrophiles examined	Percentage of cells with the following No. of granules per cell		
		Few	Moderate No.	Many
None	All	0	10	90
Type 4 streptococci	Those containing streptococci	55	35	10
" " "	Those containing no streptococci	2	13	85
Zymosan	Those containing 2–5 zymosan particles	55	38	7
"	Those containing 1 zymosan particle	17	44	39
"	Those containing no zymosan	1	11	88

examination also revealed degranulation of human phagocytes in association with ingestion of zymosan.

The degranulation phenomenon consisted of diminution in numbers of visible cytoplasmic granules, rather than in an altered appearance of all granules. Preservation of a few normal appearing granules in leucocytes engulfing large numbers of microbes provided evidence that the presence of intracellular bacteria did not alter staining properties of the granules. The possibility of granule loss by egestion or cell rupture was ruled out by several features: no cell rupture was detected in phase contrast or trypan blue studies of duplicate samples examined in the living state; degranulated stained leucocytes often showed an intact, well outlined cell border; and finally, extracellular granules were rarely seen. Vacuole formation visible in the light microscope varied considerably with different microorganisms; no correlation could be established between vacuole formation about ingested bacteria and loss of cytoplasmic granules.

DISCUSSION

It is somewhat surprising that degranulation of polymorphonuclear leucocytes following engulfment of microorganisms has not been noted previously, since many investigators have made microscopic observations of interactions between phagocytes and bacteria. Probably technical factors were largely responsible for rendering the phenomenon not readily demonstrable. Several of these technical aspects might be mentioned. First of all, the rabbit polymorphonuclear leucocyte is particularly suitable for studies on cytoplasmic granules, since in this cell the granules are relatively uniform in size and number, and are furthermore easily visualized in stained smears because of their affinity for acid dyes. Even when employing rabbit cells, however, demonstration of degranulation following phagocytosis in blood or other suspension systems is difficult, since engulfment is regularly accompanied by clumping of leucocytes and platelets. Addition of bacteria in large numbers to heparinized blood samples *in vitro* leads to changes similar to those seen *in vivo*; on mixing the white cell and platelet counts drop, and careful examination reveals large platelet-leucocyte clumps which cannot be readily studied for fine aspects of cellular morphology. Leucocytes allowed to settle on glass slides become firmly adherent yet are able to migrate and engulf particles presented to them. A suitable technique for flash drying these slide preparations then permits reliable observation of cellular morphology in relation to phagocytosis. With appropriate technique, then, the relationship between phagocytosis and degranulation becomes obvious.

From the observations made thus far, it seems that many types of bacteria or other particulate objects of size and surface properties suitable for phagocytosis lead to lysis of granules in polymorphonuclear leucocytes. Degranulation occurs only in those cells which have ingested foreign material, and it takes place with reasonable rapidity; *i.e.*, within 30 minutes. White cells which have engulfed large numbers of bacteria or zymosan show, in addition to loss of granules, a change in surface properties (clumping, rounding up) and reduction in motility, but they remain intact and viable for more than 1 hour. Whether such cells are destined to die, or whether they may recover and regenerate granules has not been determined. Since the life span of the polymorphonuclear leucocyte is probably quite short (8) and since they are end-cells incapable of division, it seems unlikely that recovery of full activity and regeneration of cytoplasmic granules will occur.

From the information in the present and the preceding report, it is possible to construct a hypothetical specialized system and sequence of events which endow the polymorphonuclear leucocyte with perhaps its most important function, namely that of killing and digesting microorganisms. These cells subsist primarily on glycolytic carbohydrate metabolism, resulting in production of large amounts of lactic acid. Present in the cytoplasm are specific granules

which contain the potent bactericidal agent phagocytin, and also several hydrolytic enzymes. These granules are lysed on exposure to a pH slightly lower than that of the normal leucocytic cytoplasm. Upon ingestion of foreign particles, glycolysis is markedly accelerated, leading to increased acid production and a fall in pH sufficient to release from the granules their content of phagocytin and digestive enzymes. Or an alternative hypothesis for granule lysis following phagocytosis might invoke vacuole formation about the engulfed material and discharge of granule contents into the vacuole upon fusion of their membranes. Current studies in collaboration with Dr. David Luck of the phagocytic process by electron microscopy have confirmed degranulation following ingestion of bacteria by polymorphonuclear leucocytes. Observations are also being made to determine whether the granules discharge their contents into the cytoplasm or into phagocytic vacuoles. In any event the polymorphonuclear leucocyte clearly is endowed with a reserve system in its granular compartment which is called into play at the time of need and as a consequence of contact with the invading microbes.

SUMMARY

A marked reduction in numbers of cytoplasmic granules in rabbit and human polymorphonuclear leucocytes takes place following ingestion of various microorganisms or of a yeast cell wall preparation. The degranulation occurs within 30 minutes of phagocytosis, and is directly related to the quantity of material engulfed. White cells completely degranulated following phagocytosis of large numbers of microorganisms remain viable for at least 1 hour.

The granules of polymorphonuclear leucocytes contain the antimicrobial agent, phagocytin, and various digestive enzymes. These substances thus are released into the cytoplasm or into vacuoles following ingestion of foreign material. The granule system and granule lysis mechanism may well play a central role in the primary function of these specialized cells; namely, that of destroying invading microorganisms.

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EXPLANATION OF PLATE 86

FIG. 1. Photographs were taken on Ektachrome type B film at a final magnification of 1080.

A. Illustrates a typical field of rabbit polymorphonuclear leucocytes (heterophiles). Note the pale blue cytoplasm and the well defined acidophilic cytoplasmic granules.

B. Shows a rabbit eosinophile in addition to the heterophilic leucocytes. The eosinophile is distinguished by its larger size, bilobed nucleus, and cytoplasmic granules which are larger and of a different hue than are those of the heterophiles.

C. Demonstrates loss of cytoplasmic granules in two rabbit polymorphonuclear white cells which have ingested chains of streptococci.

D. Illustrates degranulation of rabbit heterophiles following engulfment of zymosan. Zymosan particles appear within the cells as pale blue bodies surrounded by a large clear or slightly pink zone. The upper cell has engulfed only one zymosan particle, and shows partial degranulation, whereas the lower cell containing three zymosan particles shows marked loss of cytoplasmic granules.

E. Is a photograph of human polymorphonuclear leucocytes, one of which has ingested three zymosan bodies. Cytoplasm of the normal human granulocyte is packed with large numbers of tiny neutrophilic or faintly acidophilic particles. Marked dimininution in cytoplasm granularity is seen in the phagocyte containing zymosan.

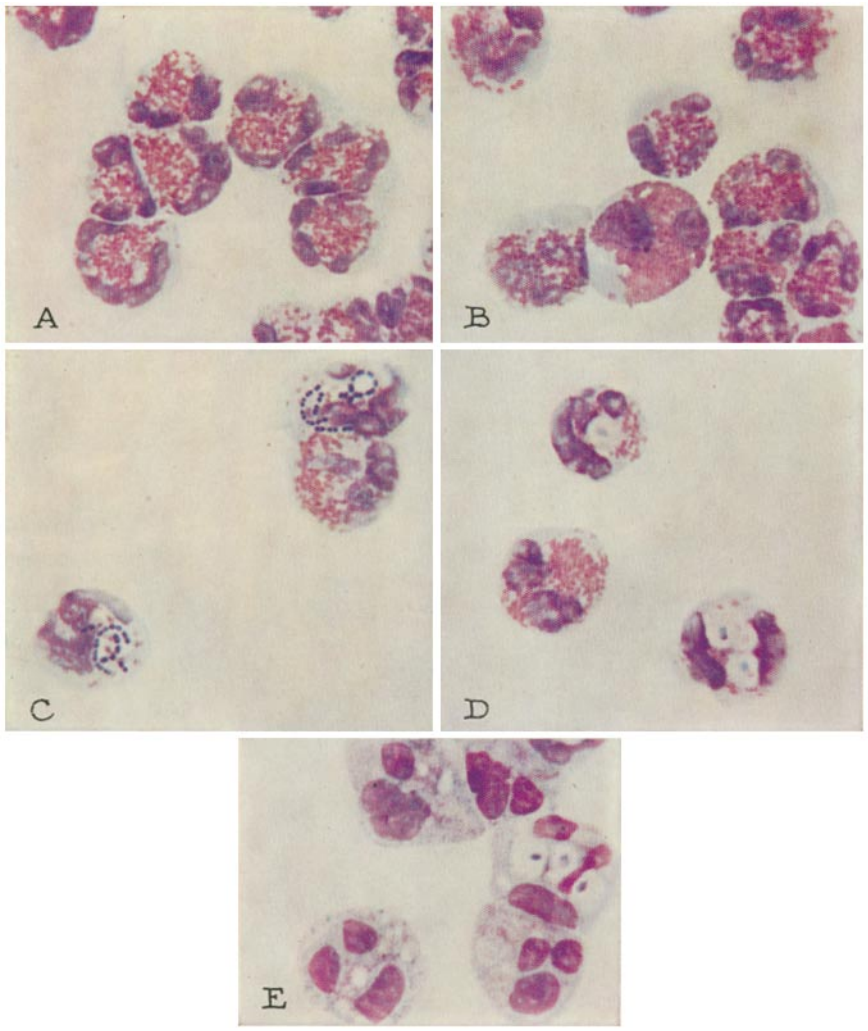

FIG. 1

(Hirsch and Cohn: Degranulation of leucocytes)