INFECTION AND IMMUNITY, Oct. 1975, p. 813-820

Vol. 12, No. 4
Printed in U.S.A.

Fate of Human Lactoferrin and Myeloperoxidase in Phagocytizing Human Neutrophils: Effects of Immunoglobulin G Subclasses and Immune Complexes Coated on Latex Beads

MARY S. LEFFELL AND JOHN K. SPITZNAGEL*

Department of Bacteriology and Immunology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

Received for publication 28 May 1975

Human neutrophils (PMN) degranulated in response to soluble human immune complexes and to myeloma proteins, including subclasses of immunoglobulin G $(IgG)_1$, IgG_2, IgG_3, and IgG_4 coated on 1.09-μm latex beads. Immunochemical measurement of lactoferrin (LF) from specific granules and myeloperoxidase (MPO) from azurophil granules showed that both classes of granule degranulated. Beads with soluble complexes of human anti-pigeon IgG-normal pigeon IgG, prepared from serum of a patient with pigeon breeders disease, induced significantly greater degranulation than did pigeon IgG-coated beads. Up to 40% of LF in the PMN degranulated during phagocytic challenge and 86% of that entered the extracellular fluid. Twenty to 30% of the MPO degranulated, but less than 50% of that entered the extracellular fluid. The degranulated LF and MPO which remained in the PMN were recovered from phagocytic vacuoles. Beads coated with purified human myeloma proteins (12 different ones, three of each subclass) induced degranulation in the order $IgG_3 > IgG_1 > IgG_2 > IgG_4$; however, these differences were found to be a function of the amount of latex ingested. Thus, the amount of degranulation was dependent more on the opsonizing capacity of the immunoglobulins rather than on their intrinsic capacities for inducing degranulation. Degranulation of both LF and MPO in response to IgG subclasses followed patterns similar to those caused by soluble immune complexes, and IgG_3 coated on beads caused degranulation equal to that caused by human complex-coated beads. Degranulation to IgG_3 and IgG_4 was uninfluenced by fresh compared with heat-inactivated human AB serum. This was true although IgG_3 beads fixed greater than sixfold more complement than did IgG_4 beads. Evidently human IgG subclasses enhance phagocytosis and degranulation of human PMN. The overwhelmingly extracellular degranulation of LF in response to various bead coatings suggest that it subserves a major portion of its role outside PMN.

in our earlier studies on degranulation of human polymorphonuclear granulocytes (PMN), rabbit immune complexes on beads induced both specific and azurophil granules to degranulate (20). The lactoferrin (LF) of specific granules (28) degranulated more extensively than the myeloperoxidase (MPO) of the azurophils (3, 28). Surprisingly, more than 90% of the degranulated specific granule contents appeared extracellularly. Only 50% of the degranulated azurophil MPO left the PMN (20; M. S. Leffell and J. K. Spitznagel, Fed. Proc. 32:291, 1973). The present experiments were intended to ask if human immune complexes produce similar effects and to determine whether different immunoglobulin subclasses influence degranulation in different ways. PMN may mediate tissue damage in several clinical and experimental inflammatory conditions. The damage presumably is due to immune complexes that trigger a sequence of events attracting PMN and causing them to release extracellularly hydrolytic enzymes and inflammatory agents (7, 8) from PMN cytoplasmic granules. PMN are especially liable to release granule enzymes after adherence to immune complexes bound to surfaces too large to be phagocytized (16).

The outcome of such studies has been taken to support the relevance of PMN degranulation for immune tissue damage; however, degranulation is also clearly related to intraleukocytic

killing of bacteria (see 26 for review). Both functions evidently relate to interactions between antibody complexed to antigen and activated complement components (9, 13, 23, 26). Yet, immune tissue damage is destructive; antimicrobial phagocytosis is usually conservative. Most studies of degranulation with PMN tend to focus on either immune tissue damage or on phagocytosis of microbe-size particles, and results are obtained as measurements of β-glucuronidase, lysozyme, or peroxidase, which are solely or largely associated with azurophil granules (2, 28). Either loss of enzymes from the cells or their retention in phagocytic vacuoles, but not both, are emphasized. We have proposed that additional insight into PMN function might be obtained if degranulation of specific and azurophil granules, both intra- and extracellularly, were studied and have reported methods for doing this (20; Leffell and Spitznagel, Fed. Proc. 32:291, 1973). It is our hope that studies of this kind will illuminate the interplay between specific and azurophil granule components and help clarify some relationships between anti-microbial phagocytosis and inflammation.

MATERIALS AND METHODS

Isolation of PMN. PMN were separated from human peripheral blood by dextran sedimentation and erythrocytes were removed by hypotonic lysis as previously described (20). Cells were washed and resuspended in TC-199 medium (Grand Island Biological Co., Grand Island, N. Y.) with 5 U of heparin per ml and 10% AB+ normal serum. The human serum had been stored in −70 C to ensure preservation of complement (C′). Cell suspensions consisted of 85 to 90% PMN and were adjusted to 3×10^7 to 5×10^7 cells/ml.

Incubations of PMN with latex beads. Polystyrene latex beads of uniform 1.09-μm diameter were otained from Dow Chemical Co., Indianapolis, Ind. Cells were incubated with polystyrene latex beads which had been coated with various purified immunoglobulins or immune complexes (see below). Latex was added to a final concentraton of 2 mg/ml. Incubation was for 1 h at 37 C with gentle shaking.

Adsorption of immunoglobulins and immune complexes to latex beads. The immune globulins and complexes were adsorbed to the latex beads according to van Oss and Singer (25). Beads were mixed with various proteins and were incubated for 1 h at 37 C, washed free of nonadsorbed protein by five washes with Krebs Ringer phosphate buffer, pH 7.4, and then resuspended in the same buffer to a 10% suspension. Twelve purified myeloma proteins of subclasses of immunoglobulin G $(IgG)_1$, IgG_2, IgG_3, and IgG_4 (three of each subclass) were generously donated by W. Yount, Department of Medicine, University of North Carolina Medical School, Chapel Hill; the mean milligrams of protein adsorbed per milligram of latex beads (assayed by the Lowry method [21]) were 0.015, 0.019, 0.027, and 0.011, respectively, for IgG_1 through IgG_4.

Human anti-pigeon IgG-pigeon IgG complexes. Serum from a patient with pigeon breeders disease with a high titer of precipitating antibody to pigeon IgG was kindly supplied by R. Wistar of the National Naval Medical Center, Bethesda, Md.; quantitative precipitation of this serum with the pigeon IgG allowed the preparation of precipitates at equivalence. After thorough washing, these precipitates were dissolved by addition of excess antigen. Single radial immunodiffusion analysis (22) of the prepared complexes showed the human antibodies to pigeon IgG were primarily of the IgG class, and that the molar antibody/antigen ratio was 0.5. Beads coated with complexes carried 0.0095 mg of protein/mg of latex. Beads coated with pigeon IgG-human antibody complexes carried 0.01 mg of protein/mg of latex.

Isolation of extracellular fluid. After the PMN were offered a phagocytic challenge and had been incubated for the desired time, we centrifuged the suspension at $126 \times g$ for 10 min and removed the supernatant fluid which contained uningested beads. The cell button was then washed. Experiment showed that negligible PMN granule markers and negligible (lactate dehydrogenase) appeared in the washings. The supernatant extracellular fluid was cleared of beads and assayed for granule markers and for LDH. The results were computed in absolute terms, i.e., micrograms of granule substance excreted per milligram of latex ingested. They were also calculated as percentage excretion of the substance present in the original cell suspension with which the experiment was begun (see Fig. 1 and 2). The cell pellet was used for the isolation of phagocytic vacuoles.

Isolation of phagocytic vacuoles. Vacuoles formed about latex beads in PMN were separated from cell homogenates on discontinuous sucrose gradients. Washed cell pellets with ingested latex were homogenized in 30% (net/vol) sucrose by 24 to 30 passes in a chilled Dounce homogenizer with a tight pestle. The cell homogenates were brought to 50% sucrose by the addition of concentrated sucrose. Discontinuous gradients were formed by overlaying 5 ml of the cell homogenate in 50% sucrose with 5 ml of 40% and 7 ml of 30% sucrose solutions. Resolution of the gradients in a SW27.1 rotor at $100,000 \times g$ for 1 h in a Beckman Spinco L2-65B centrifuge resulted in the flotation of the latex bead-filled vacuoles to the top of the gradient, while cellular debris and soluble proteins remained at the tube bottom in the loading zone. We have shown that the isolated bead fractions are membrane rich (20; Leffell and Spitznagel, Fed. Proc. 32:291, 1973). Samples of the original cell suspension before phagocytosis and aliquots of the incubation mixtures were saved and analyzed with all gradient fractions. To determine the degree of degranulation into phagocytic vesicles, the quantities of the granule marker proteins, LF and MPO, associated with the vesicles were compared with the amounts originally in the cell suspension. Granule protein released into the medium during phagocytosis was compared with the proteins in the original

cell suspension. Total recoveries of LF, MPO, and LDH were calculated as follows: for sucrose density gradients, the sum of LF or MPO in gradient fractions/LF or MPO in cell homogenate applied to gradient ×100; for cells and incubation medium after separation from each other, the sum of LF, MPO, or LDH in medium and in cell button from medium/LF, MPO, or LDH in incubation mixture ×100.

Immunochemical determinations of LF and MPO. LF and MPO were used, respectively, as markers for the specific and azurophil granule classes. With specific antisera and purified LF and MPO as standards, these two marker proteins could be quantitated in PMN subcellular fractions by single, radial immunodiffusion (16, 18, 20, 28) as described by Mancini et al. (22). To obtain full recovery of both proteins, Mancini plates were prepared in 0.01 M phosphate buffer, pH 8.6, with 1 M NaCl. All PMN vacuole and subcellular fractions were extracted in 0.1% cetyl trimethylammonium bromide in the same phosphate buffer. This was necessary because the granule components in the cell homogenates were latent, i.e., undetectable without detergent extraction and therefore membrane bound. Aliquots of the incubation medium freed of cells were concentrated twofold and were tested without cetyl trimethylammonium bromide extraction. Preliminary experiments showed that granule components in incubation medium were not latent. All determinations were done in duplicate.

Enzymatic and chemical determinations. *p*-Dioxane extraction of latex from PMN fractions, as described by Werb and Cohn (32), were used to quantitate phagocytosis. LDH, assayed according to Bergmeyer et al. (4), was used as the criterion for judging loss of enzymes that could have occurred by cell lysis. Allowance was made for LDH contributed by the human serum. Latex turbidity was cleared from samples before spectrophotometric readings by filtration through 0.8-μm membrane filters (Millipore Corp.). The ability of the myeloma protein-coated beads to fix C′ was determined using guinea pig C′ in a standard sheep erythrocyte hemolysin procedure (6).

RESULTS

The results of four experiments in which beads coated with soluble complexes of human, anti-pigeon IgG-pigeon IgG were compared with those coated only with antigen are summarized in Fig. 1. In response to both kinds of coating, the bulk of the degranulated LF entered the extracellular space. Of the LF initially in the specific granules, 43% degranulated in response to complexes and of that 86% left the cells; 14% remained in the phagocytic vacuoles. Of the MPO initially in the azurophil granules, 32% degranulated and 45% of that left the cells with 54% remaining the vacuoles. Complexes compared with antigen increased overall degranulation of LF about 50% whereas they increased that of MPO 60%.

Complexes caused greater increases in degranulation of LF into extracellular fluid and of MPO into vacuoles than any other coating on beads. LF which degranulated and remained in phagocytic vacuoles, and MPO which entered extracellular fluid were increased only slightly by complexes compared with antigen. Loss of LDH from cells was less than 5% and indicated that LF and MPO losses were not caused by cytotoxicity.

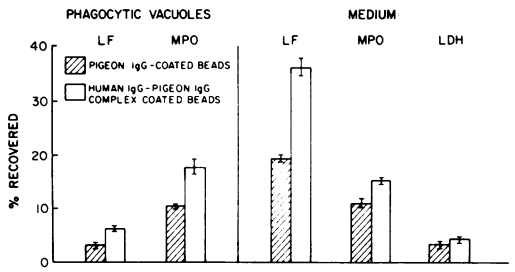

FIG. 1. *Transfer of PMN granule components into phagocytic vacuoles and extracellular medium induced by latex beads coated with human anti-pigeon IgG-pigeon IgG complexes or uncomplexed pigeon IgG. Percentage recovered is the proportion of substance recovered in phagocytic vacuoles or in extracellular medium expressed as percentage of the substance present in the PMN suspension before phagocytic challenge. Histograms represent mean percentages with standard error of the mean indicated by brackets for four experiments. Overall recoveries were calculated as shown. Sucrose density gradients = sum of LF or MPO recovered from sucrose density gradient fractions/LF or MPO applied to gradients in cell homogenate ×100. Extracellular medium plus cell button = sum of LF, MPO, or LDH in medium and cell button both separated from reaction mixture/LF, MPO, or LDH in reaction mixture prior to separation. Sucrose density gradients: LF, 96.6 ± 1.3, 90.6 ± 2.3; MPO, 91.3 ± 0.7, 90.5 ± 2.7. Extracellular medium plus cell button: LF, 83.2 ± 2.3, 85.4 ± 1.6; MPO, 84.3 ± 2.1, 93.0 ± 7.9; LDH, 103.2 ± 2.3, 94.4 ± 4.4.*

Upon microscopic examination of the PMN after phagocytosis, it appeared that the PMN took up more of the complex-coated beads than they did of the antigen-coated beads; spectrophotometric measurements of latex uptake confirmed this. Therefore, we attempted to correlate phagocytic uptake with the subsequently induced degranulation. The latex taken up by the cells was quantitated by *p*-dioxane extraction of cell homogenates. The total micrograms of LF and MPO released into both vacuoles and medium were then expressed in terms of the milligrams of latex ingested (Table 1). The complexes induced greater releases of LF per milligram of ingested latex. Complex-coated beads induced 18% increase in degranulation of LF into phagocytic vacuoles and a 40% increase in

TABLE 1. *Phagocytic uptake with degranulation stimulated by human IgG:pigeon IgG complex-coated beads compared with pigeon IgG-coated beads*[a]

Phagocytic stimulus	Latex uptake	Vacuoles		Medium	
		LF	MPO	LF	MPO[b]
Human IgG:pigeon IgG complexes	0.8 ± 0.01[c]	3.3 ± 0.1[d]	10.3 ± .58	33.4 ± 0.8	11.8 ± 0.5
Pigeon IgG	0.6 ± 0.04	2.8 ± 0.1	8.7 ± .58	23.2 ± 1.2	11.4 ± 0.5

[a] Mean ± standard error of the mean for four experiments are given.
[b] LDH in medium <2% total in PMN.
[c] Figures are for milligrams of latex ingested/10^7 cells.
[d] Values were calculated by the formula: (total micrograms of LF or MPO released/10^7 cells)/(total milligrams of latex ingested/10^7 cells).

degranulation of LF into the extracellular space compared with that caused by uncomplexed antigen on beads. Degranulation of MPO into phagocytic vacuoles was increased by 18%.

Beads with human myeloma IgG proteins of different subclasses. To investigate the effects of IgG subclasses in stimulating granule enzyme release, we coated latex beads with purified myeloma proteins of different IgG subclasses. Singer has shown that the binding of these subclasses to polystyrene latex confers upon them properties of aggregated immunoglobulins (see reference 17 in 25). As aggregated immunoglobulins fix complement (1), we decided first to determine the C′ binding ability of the coated beads as a measure of their biological activity. The results of the C′ fixation titration are shown in Table 2. Twelve different myeloma proteins, three of each subclass, were tested. It can be seen that they did bind C′. Interestingly, the ability to bind C′ was dependent upon IgG subclass, with C′ binding decreasing in the order $IgG_3 > IgG_1 > IgG_2 > IgG_4$. The order of subclass C′ binding agreed with that reported by Augener et al. (1) for monomeric immunoglobulins.

Figure 2 demonstrates the percentages of LF and MPO degranulation stimulated by the immunoglobulin-coated beads. As a general observation, it appeared that the myeloma proteins stimulated degranulation in the same order that they bound complement. IgG_3 generally induced only slightly greater percentages of release than IgG_1 or IgG_2, but obviously greater release than IgG_4.

Further analysis, however, showed that the degree of degranulation correlated with phagocytic uptake. Table 3 shows these results in terms of latex ingested per 10^7 PMN. It can be seen that PMN tended to take up the myeloma protein-coated beads in the order in which the beads fixed C′, i.e., $IgG_3 > IgG_1 > IgG_2 > IgG_4$. However, the differences induced in degranulation per milligram of ingested latex were relatively insignificant. Once latex was phagocytized, the quantity ingested may have been the principal determinant for the degree of degranulation. The IgG subclass appeared to influence the uptake of latex.

TABLE 2. *C′ fixation by latex bead-adsorbed myeloma proteins of different IgG subclasses*

Subclass			
IgG_1	IgG_2	IgG_3	IgG_4
421.7[a]	322.3	>939	133.8
633.0	301.5	>750	105.2
581.6	313.2	>600	121.7

[a] Results expressed as 50% units of C′ fixed/milligrams of protein.

Effects of fresh serum. Since beads coated with different IgG subclasses fixed different amounts of complement, we wished to see if the fresh serum was essential to the system. In Table 4 the uptake of and degranulating effects of IgG_3-coated and IgG_4-coated beads were compared in the presence of fresh and heated serum. Fresh compared with heat-inactivated serum appeared to have no systematic influence on degranulation.

DISCUSSION

The points of principal interest in this, as well as in our earlier paper (20), are the predominately extracellular degranulation of LF and the way this contrasts with degranulation of MPO of human PMN. That nearly 90% of the degranulated LF leaves the cell whereas less than 50% of degranulated MPO leaves suggests these are independent events (20). In the same context it is notable that, even with vigorous stimulus, overall degranulation of LF may achieve 60% whereas degranulation of MPO seldom exceeds 30%. Since homogenization may rupture some phagocytic vesicles the 60

and 30%, respectively, may represent minimal estimates. Nevertheless the results are in agreement with morphological studies in which electron micrographs show rabbit PMN azurophils

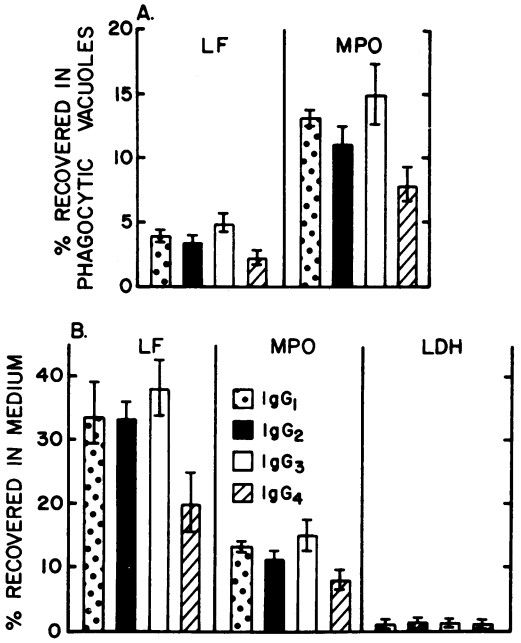

FIG. 2. *Transfer of PMN granule components into phagocytic vacuoles and extracellular medium induced by latex beads coated with human myeloma proteins of different IgG subclasses. Percentage recovered in phagocytic vacuoles and percentage recovered in extracellular medium are calculated as in Fig. 1. Histogram as for Fig. 1. Overall recoveries from sucrose density gradients and overall recovery from extracellular medium plus cell buttons are calculated as for Fig. 1. Sucrose density gradients: LF, 91.5 ± 3.6, 91.5 ± 2.7, 93.2 ± 1.3, 86.5 ± 3.6; MPO, 88.8 ± 3.1, 92.5 ± 2.8, 92.2 ± 2.3, 83.5 ± 1.9. Extracellular medium plus cell button: LF, 87.7 ± 3.6, 91.0 ± 2.4, 94.2 ± 2.8, 89.0 ± 3.0; MPO, 85.5 ± 4.9, 88.5 ± 2.7, 96.8 ± 2.9, 87.7 ± 7.7; LDH, 86.3 ± 2.0, 87.5 ± 2.0, 91.5 ± 4.6, 84.8 ± 3.3.*

TABLE 4. *Effects of fresh human serum compared with heat-inactivated serum on phagocytosis of latex beads coated with IgG_3 and IgG_4*

Reaction mixture	Latex ingested[a]	Protein released[b]			
		Phagosomes		Medium[c]	
		LF	MPO	LF	MPO
IgG_3+C'[d]	0.45	2.9	7.8	59.6	11.6
IgG_3+ΔC'[e]	0.38	2.9	6.6	59.5	12.9
IgG_4+C'	0.29	3.4	7.9	53.4	14.1
IgG_4+ΔC'	0.39	2.6	6.2	44.4	12.1

[a] Milligrams of latex/10^7 PMN.
[b] Micrograms of protein released per milligram of ingested latex.
[c] LDH in medium <2% total in PMN in all tubes.
[d] C', Fresh human AB serum 10%.
[e] ΔC', Fresh human serum heated at 56 C for 20 min, 10%.

degranulate to a lesser extent (2) than specifics. In any event underestimation of the granule components in phagolysosomes would not vitiate the fact that considerable amounts of LF and substantially less MPO may leave the cell during phagocytosis of coccus-sized spheres.

Evidently LF, which is packaged in specific granules as an apoprotein with strong iron and copper complexing capacities, could mediate some important function outside PMN either in extracellular space or even in another cell. LF released from PMN could, according to Van Snick et al. (31), account for the hypoferremia of acute inflammation. They have shown, moreover, that iron bound to LF is taken up by macrophages. Thus it is conceivable LF could mediate antimicrobial or other actions in cells other than PMN. Our results do not necessarily supercede but may extend the earlier concept that LF performs intraleukocytic killing. This earlier concept arose in relation to the discovery of LF in PMN cytoplasmic granules (discussed in references 19, 20, 26, and 38) and was

TABLE 3. *Comparison of phagocytic uptake and degranulation stimulated by latex beads coated with myeloma proteins of different IgG subclasses*[a]

Subclass	Latex uptake	Vacuoles		Medium[b]	
		LF	MPO	LF	MPO
IgG_1	0.37 ± 0.05[c]	3.4 ± 0.6[d]	13.3 ± 2.4	53.1 ± 7.0	17.0 ± 1.7
IgG_2	0.33 ± 0.04	3.4 ± 0.4	11.1 ± 0.5	60.5 ± 2.5	22.1 ± 2.0
IgG_3	0.40 ± 0.07	4.5 ± 1.0	14.1 ± 2.8	65.2 ± 9.7	22.2 ± 2.0
IgG_4	0.22 ± 0.03	3.5 ± 0.7	11.9 ± 2.7	52.4 ± 13.4	19.9 ± 2.8

[a] Mean ± standard error of the mean for four experiments are given.
[b] LDH in medium <5% of total in PMN in all tubes.
[c] Figures are for milligrams of latex ingested/10^7 cells.
[d] Values were calculated by the formula: (total micrograms of LF or MPO released/10^7 cells)/(total milligrams of latex ingested/10^7 cells).

supported by observations on a patient with absence of LF and specific granules from his PMN. His PMN phagocytized adequately but had reduced intraleukocytic killing capacity for gram-negative rods and enterococci (J. K. Spitznagel, M. R. Cooper, C. E. McCall, L. R. DeChatelet, and I. R. Welch, Proc. Am. Soc. Clin. Invest., Abstr. 93a, 1972). It should be emphasized that, since PMN retain definite amounts of LF in their phagolysosomes, intraleukocytic as well as extraleukocytic roles are possible for this protein. Unfortunately much remains to be learned about the antimicrobial action of LF and inferences concerning its locus of action cannot be drawn from its mechanism of action.

LF, according to Masson (references in 20 and 28), may possess primary antimicrobial activity. Gladstone (10) has stated its primary antimicrobial capacity is relatively weak but he suggested that LF could bind iron and protect antimicrobial action of PMN from inhibition by this metal. In a recent abstract it has been suggested that iron complexed to LF is an effective peroxidase which could function as a microbicide (R. S. Dobrin, A. F. Michael, J. Hareman and B. Holmes, Fed. Proc. 34:4680, 1975).

Actually it is possible that any of these actions could occur extra- as well as intracellularly. For example it has been emphasized that H_2O_2 is excreted by PMN (R. K. Root, N. Oshuno, and B. Chance, Clin. Res. 21:970, 1973). Thus an extracellular peroxidatic mechanism could be supported as discussed below. Experiments are needed to identify more precisely the nature and locus of action of various antimicrobial substances of PMN.

Since the antimicrobial action of MPO appears to reside in its enzymatic enhancement of H_2O_2 in the presence of Cl^- (reference in 26), the fact that a smaller proportion of MPO than of LF degranulates during phagocytosis in no way detracts from the postulated role of MPO in intraleukocytic killing. The amounts deposited in phagocytic vesicles would probably be sufficient to promote antimicrobial action. Moreover, some of the MPO leaves the cell and H_2O_2 is present outside the cell (Root, Oshuno, and Chance, Clin. Res. 21:970, 1973); hence, it could be that a zone of antimicrobial action is set up in the vicinity of the PMN.

The present work showed that, within 60 min after phagocytosis is initiated, LF is degranulated to a greater extent than MPO. Remarkably, 86% of the LF which degranulated left the cell and was exocytosed. That this exocytosis occurred in response to human immune complexes or to human IgG subclasses coated on 1.09-μm latex beads satisfied a goal of this work. We had previously shown that this pattern of degranulation occurred among human PMN in response to rabbit immune complexes (20) and wished to know if human immunoglobulin would have like action.

These experiments revealed for the first time with biochemical methods the fate of human PMN LF and specific granules in response to human immunoglobulin. As we discussed previously (20), Bainton's studies of ultrastructure (2) clearly showed that degranulation of specific granules in rabbit PMN precedes that of azurophil PMN. However, biochemical studies done by others on degranulation of specific granules in human PMN are difficult to interpret because alkaline phosphatase and lysozyme were the only markers used that might have indicated degranulation of specifics (9). Unfortunately alkaline phosphatase provides no marker for specific granules in human PMN (19, 28), and lysozyme is an ambiguous marker for specific granules in human PMN, for it is distributed 50:50 between them and azurophil granules.

If our quantitative results with degranulation of azurophil granules are compared with results of others, it is clear that there are considerable similarities. Both immune complexes (12–15, 30, 32) and myeloma proteins of subclasses IgG_1, IgG_2, IgG_3, and IgG_4 coated on surfaces (15) stimulate exocytosis of MPO from azurophil granules. We found degranulation to immune complexes exceeded that to antigen alone. In fact only antigen-coated beads induced less degranulation than did complexes. In our previous work rabbit antibody was used with bovine serum albumin as antigen and neither antibody nor antigen alone induced as much or as rapid degranulation as the complexes. In the present work a patient with pigeon breeders disease furnished the antibody. Normal pigeon IgG comprised the antigen. Thus it seems that human IgG coated on beads has biological properties for human PMN which is not possessed by heterologous immunoglobulins except when they are complexed to antigen.

In our experiments, degranulation stimulated by human immune complexes and IgG_3 appeared to surpass that due to pigeon IgG or human myeloma proteins other than IgG_3. However, when the degranulation was normalized by calculating the amount of MPO degranulated per milligram of latex ingested, none of the beads coated with myeloma proteins enjoyed a substantial advantage over each other. Azurophil degranulation appeared heavily influenced by the load of latex picked up by the cells. This was different from our results with human or rabbit immune complexes where de-

granulation 60 min after initiation of phagocytosis seemed more related to the presence of complexes on the beads. Insofar as our results can be compared with those of Henson (13, 14), they are similar and can be interpreted to show that different IgG subclasses differed little or not at all in their degranulating effects on human PMN.

The present experiments were designed, as were our earlier ones, to maximize phagocytosis and degranulation (20) by insuring that the amounts of latex beads offered to the PMN never could be limiting for phagocytosis. Beads were always present in excess. The amounts of latex apparently ingested by the cells seemed large, possibly amounting to 60 beads/cell in some instances. Others have reported comparable particle uptake with latex beads of this size and PMN in suspension (17, 27) as well as with starch particles and PMN in monolayers (24). Although we seem to have achieved a maximal phagocytic stimulus, our results still fell short of complete degranulation. As we discussed above, homogenization could have disrupted some phagocytic vesicles and a few vesicles could have been trapped with the cell debris. This would have caused us to underestimate the degranulation into the vesicles and in that way to underestimate the overall degranulation. However, our failure to achieve total degranulation is not entirely artifact. It reflects the results of morphological studies of others. Moreover our experiments were limited to 60 min. It is entirely possible that longer periods of phagocytic stimulus would have resulted in more nearly complete degranulation.

Because phagocytosis was so massive in these experiments, it might be wondered if much degranulation to the extracellular compartment occurred just because many beads could not be completely engulfed. In other words, were the results in part caused by effects comparable to those seen with completely nonphagocytosable surface (13–15). Perhaps some such effect was present; however, the substantial differences between degranulation of specific compared with azurophil granules suggests that closure of substantial numbers of vacuoles took place. Moreover, in our earlier work (20), kinetic studies showed that such differences developed as early as 15 min after phagocytosis was begun, a time at which maximal phagocytosis was not yet achieved. It should be noted that work from several laboratories implies that substances can escape from nascent vesicles (2, 20; Root et al., Clin. Res. 21:970, 1973) and channels for this have been shown (5). Finally the minimal losses of LDH from cells showed that exocytosis and not cell damage was likely responsible for the loss of LF and MPO from the cells.

It has been suggested, based on experiments in which cytochalasin B-poisoned PMN were stimulated with C5a, that complement components are involved in degranulation (11). In experiments where we intentionally sought to reduce degranulation by leaving out complement no significant change was noted. This was in agreement with the results of Henson et al. with myeloma protein (15). This does not mean C′ would be unnecessary in other models of phagocytosis. In fact, from results of others, it seems likely that in certain systems not just IgG (23) but C′ components (13, 18, 26, 29) influence rates of phagocytosis. Whether they are directly involved in degranulation remains to be seen.

ACKNOWLEDGMENTS

This work was supported by Atomic Energy Commission Grant AI-(40-1)-3628 and by Public Health Service Grant AI02430 from the National Institute of Allergy and Infectious Diseases. M. S. L. was the recipient of an NDEA Title IV Fellowship.

LITERATURE CITED

1. Augener, W., H. M. Grey, N. R. Cooper, and J. J. Muller-Eberhard. 1971. The reaction of monomeric and aggregated immunoglobulins with C'1. Immunochemistry 8:1011–20.
2. Bainton, D. F. 1973. Sequential degranulation of the two types of polymorphonuclear leukocyte granules during phagocytosis of microorganisms. J. Cell Biol. 58:249–64.
3. Bainton, D. F., J. L. Ullyot, and M. G. Farquhar. 1972. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. Origin and content of azurophil and specific granules. J. Exp. Med. 134:907–34.
4. Bergmeyer, H., E. Bernt, and B. Hess. 1963. Lactic dehydrogenase, p. 736–743. *In* H. U. Bergmeyer(ed.), Methods of enzymatic analysis. Academic Press Inc., New York.
5. Briggs, R. T., M. L. Karnovsky, and M. J. Karnovsky. 1975. Cytochemical demonstration of hydrogen peroxide in polymorphonuclear leukocyte phagosomes. J. Cell Biol. 64:254–260.
6. Campbell, D. H., J. S. Garvey, N. E. Cremer, and D. H. Sussdorf. 1963. Immune hemolysis, p. 172–179. *In* Methods in immunology. W. A. Benjamin, Inc., New York.
7. Cochrane, C. G. 1969. Immunologic tissue injury mediated by neutrophilic leukocytes. Adv. Immunol. 9:97–162.
8. Cochrane, C. G., and D. Koffler. 1973. Immune complex disease in experimental animals and man. Adv. Immunol. 16:185–264.
9. Estensen, R. D., J. G. White, and B. Holmes. 1974. Specific degranulation of human polymorphonuclear leukocytes. Nature (London) 248:347–348.
10. Gladstone, G. P. 1973. The effect of iron and hematin on the killing of staphylococci by rabbit polymorphonuclear leukocytes. Contrib. Microbiol. Immunol. 1:222–243.
11. Goldstein, I. M., M. Brai, A. G. Osler, and G. Weissman. 1973. Lysosomal enzyme release from human leukocytes: mediation by the alternate pathway of complement activation. J. Immunol. 111:33–37.

12. Hawkins, D., and S. Peeters. 1971. The response of polymorphonuclear leukocytes to immune complexes *in vitro*. Lab. Invest **24**:483–91.
13. Henson, P. M. 1971. The immunologic release of constituents from neutrophil leukocytes. I. The role of antibody and C′ on non-phagocytosable surfaces or phagocytosable particles. J. Immunol. **107**:1535–46.
14. Henson, P. M. 1971. The immunologic release of constituents from neutrophil leukocytes. II. Mechanisms of release during phagocytosis and adherence to non-phagocytosable surfaces. J. Immunol. **107**:1547–57.
15. Henson, P. M., H. B. Johnson, and H. L. Spiegelberg. 1972. The release of granule enzymes from human neutrophils stimulated by aggregated immunoglobulins of different classes and subclasses. J. Immunol. **109**:1182–92.
16. Himmelhoch, S. R., W. H. Evans, M. G. Mage, and E. A. Peterson. 1969. Purification of myeloperoxidases from the bone marrow of the guinea pig. Biochemistry **8**:914–21.
17. Kvarstein, B. 1969. The effect of temperature, metabolic inhibitors, and EDTA on phagocytosis of polystyrene latex particles by human leucocytes. Scan. J. Clin. Lab. Invest. **24**:271–277.
18. Lay, W. H., and V. Nussenweig. 1968. Receptors for complement on leukocytes. J. Exp. Med. **128**:991–1007.
19. Leffell, M. S., and J. K. Spitznagel., 1972. Association of lactoferrin with lysozyme in granules of human polymorphonuclear leukocytes. Infect. Immun. **6**:761–765.
20. Leffell, M. S., and J. K. Spitznagel. 1974. Intracellular and extracellular degranulation of human polymorphonuclear azurophil and specific granules induced by immune complexes. Infect. Immun. **10**:1241–1249.
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
22. Mancini, G., A. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry **2**:235–254.
23. Messner, R. P., and J. Jelinek. 1970. Receptors for human gammaglobulin on human neutrophils. J. Clin. Invest. **49**:2165–2171.
24. Michell, R. H., S. J. Pancake, J. Noseworthy, and M. L. Karnovsky. 1969. Measurement of rates of phagocytosis. The use of cellular monolayers. J. Cell. Biol. **40**:216–224.
25. Oss, C. J. van, J. M. Singer. 1966. The binding of immune globulins and other proteins by polystyrene latex particles. J. Reticuloendothel. Soc. **3**:29–40.
26. Quie, P. 1972. Disorders of phagocytosis. Curr. Probl. Pediatr. **11**:3–54.
27. Roberts, J., and J. K. Quastel. 1963. Particle uptake by polymorphonuclear leukocytes and Ehrlich ascites-carcinoma cell. Biochemistry **89**:150–156.
28. Spitznagel, J. K., F. G. Dalldorf, M. S. Leffell, J. D. Folds, I. R. H. Welsh, M. H. Cooney, and L. E. Martin. 1974. Character of azurophil and specific granules purified from human polymorphonuclear leukocytes. Lab. Invest. **30**:774–785.
29. Stossel, T. P. 1973. Quantitative studies of phagocytosis. Kinetic effects of cations and heat-labile opsonin. II. J. Cell Biol. **58**:346–356.
30. Taichman, N. S., W. Pruzanski, and N. S. Ranadive. 1972. Release of intracellular constituents from rabbit polymorphonuclear leukocytes exposed to soluble and insoluble immune complexes. Int. Arch. Allergy **43**:182–195.
31. Van Snick, J. L., P. L. Masson, and J. F. Heremans. 1974. The involvement of lactoferrin in the hyposidermia of acute inflammation. J. Exp. Med. **140**:1068–1084.
32. Werb, Z., and Z. A. Cohn. 1971. Plasma membrane synthesis in the macrophage following phagocytosis of polystyrene latex particles. J. Biol. Chem. **247**:2439–2446.