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Intracellular and Extracellular Degranulation of Human Polymorphonuclear Azurophil and Specific Granules Induced by Immune Complexes

MARY S. LEFFELL AND JOHN K. SPITZNAGEL

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

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The nature of the phagocytic stimulus determines the degree of degranulation. Specifically, immune complexes induced more degranulation than did antigen or antibody alone. The release of constituents from the two granule classes represented independent phenomena. Relatively more of the specific granule proteins were released into the medium during phagocytosis, whereas more of the azurophil proteins were associated with the phagosomes.

Since the cytoplasmic granules of neutrophils (PMN) contain both antimicrobial compounds (10, 27, 48) and possible mediators of immune tissue injury (11, 25), there has been much recent interest in degranulation. One approach to studying degranulation has been to isolate developing phagocytic vacuoles and another has been to measure extracellular release of granule enzymes during phagocytosis. In theory, if cells phagocytize indigestible particles of sufficiently low density, careful homogenization of cell suspensions followed by density gradient centrifugation should allow the separation of membrane-enclosed vacuoles. Such an approach has been used with emulsified oil droplets in PMN (37) and with polystyrene latex beads in amoebas (44), macrophages (43), and PMN (J. Hawiger, S. Timmons, and M. G. Koenig, Clin. Res. **20**:82). Stimuli such as bacteria (47) and immune complexes (20-23, 39, 42) have also been used to follow the degranulation process, No studies, however, have combined measurements of degranulation into both phagosomes and extracellular medium. And although it is now generally agreed that there are two major granule classes in human and rabbit PMN (2, 4, 6), no one has studied biochemically the simultaneous degranulation of these classes.

We have previously reported the localization of the protein lactoferrin (LF) within the specific granules of human PMN (28); myeloperoxidase (MPO) is known to be included in the azurophil granule class (6, 35). We have now developed a method for isolating phagocytic vacuoles from human PMN using polystyrene latex beads coated with heterologous proteins and immune complexes, and, by using immunochemical assays for the two granule proteins LF and MPO, we have been able to follow degranulation of the two granule classes into both phagosomes and extracellular medium in response to stimuli of different magnitudes.

(This paper is taken in part from a doctoral dissertation submitted by M. S. L. in partial fulfillment of the requirements for the Ph.D. degree to the University of North Carolina at Chapel Hill.)

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MATERIALS AND METHODS

Isolation of PMN. Human PMN were obtained from peripheral blood by dextran sedimentation as previously described (28). After removal of the sedimented erythrocytes, the leukocytes were removed from the dextran by centrifugation at 126 × *g*. Contaminating erythrocytes were then removed by hypotonic lysis (15). Finally, the leukocytes were washed once with tissue culture medium (TC-199, Grand Island Biological Co., Grand Island, N.Y.) with 5 U of heparin/ml, and the resulting cell suspensions contained at least 90% PMN.

Preparation of latex beads for phagocytosis. Polystyrene latex beads of a uniform 1.099-μm diameter were obtained from Dow Chemical Co., Indianapolis, Ind. These beads were dialyzed for several days against distilled water or were washed repeatedly by centrifugation before use. In the first series of experiments, beads coated with bovine serum albumin (BSA) were added as the phagocytic stimulus. In later experiments, the beads were coated with either BSA, rabbit anti-BSA, or BSA-anti-BSA complexes. The principles of van Oss and Singer were used in such labelings (40). For BSA and the anti-BSA serum, bead labeling was accomplished by incubating mix-

tures of beads and proteins in a slightly alkaline (pH 7.4) solution with gentle heat (37 C). After at least 1 h of incubation, the beads were washed five times with Krebs-Ringer phosphate buffer, pH 7.4, and resuspended to their original volume in the same buffer. BSA-anti-BSA complexes were prepared by absorbing the anti-BSA serum with previously BSA-coated beads. Protein absorbed to the beads was measured by the method of Lowry et al. (29). In these experiments there were approximately 800 μg of both the BSA and the anti-BSA serum and approximately 1,400 μg of the complexes coated on the beads contained in 1 ml of a 10% latex solution. Immune complex formation on the beads was indicated by the ability of the beads to fix complement. This assay was a standard sheep erythrocyte-hemolysin system as described by Campbell (9). A purified immunoglobulin G (IgG) fraction from the rabbit anti-BSA serum was used for some experiments. This IgG fraction was isolated by affinity chromatography using BSA coupled to CNBr-activated sepharose (12). The purity of the IgG fraction was checked against whole rabbit serum by immunoelectrophoresis. By absorbing dilutions of the anti-BSA IgG fraction with previously coated BSA beads, IgG could be complexed with BSA on the beads in molar ratios of 0.02, 0.4, 0.8, 1.0, and 1.2 For the time course study, such complex-coated beads with a molar ratio of 0.3 were used. For uptake studies beads were coated with ^{125}I-labeled BSA and ^{125}I-labeled BSA-anti-BSA complexes. ^{125}I (ICN-Tracerlab, Irvine, Calif.) was coupled to crystalline BSA by the MacFarlane monochloride iodination technique (30). In no instance did the latex beads show gross or microscopic agglutination.

Incubations. The PMN leukocytes were suspended in TC-199 with 5 U of heparin/ml and 10% AB+ normal human serum to concentrations of 3×10^7 to 5×10^7 leukocytes/ml. The human serum either was fresh or had been stored at −70 C to preserve complement activity. The cells were incubated in siliconized glass Erlenmeyer flasks at 37 C with gentle shaking. Latex beads were added at 0 time to a final concentration of 2 mg/ml or 100 beads/leukocyte. Samples of the original leukocyte suspension were saved for analysis before phagocytosis and degranulation. The cells were incubated for 1 h, after which time phagocytosis was stopped with 1 volume of ice-cold Gey balanced salt solution made according to Dresser and Wortis (13). Cells were removed by centrifugation at $126 \times g$ for 5 min, and samples of the incubation medium were saved. The cells were washed three times in Gey solution to remove uningested beads.

Homogenization of PMN and gradient separation of latex-bead phagolysosomes. The washed PMN pellets were resuspended in freshly prepared 30% sucrose (wt/vol), 4 volumes of sucrose being added to 1 volume of cells. The suspensions were homogenized in a chilled Dounce homogenizer with a tight pestle at 4 C. Usually 25 to 30 passes were required to obtain 90% or greater breakage (checked microscopically). Cell homogenates were then brought to 50% (wt/vol) sucrose by the addition of concentrated sucrose. The gradients were formed by overlaying 5 ml of the cell homogenate with 5 ml of 40% sucrose and then with 7 ml of 30% sucrose (Fig. 1). The gradients were then resolved in an SW27.1 rotor at 100,000 × *g* for 1 h in a Beckman Spinco L2-65B centrifuge, and six fractions were collected as shown. The pellet, which contained the nuclear debris, was rehomogenized in a Tenbroek homogenizer to assure complete redispersement.

Calculations of marker protein transfer into phagosomes were based on the total activity recovered in gradients, whereas calculation of marker protein release into the medium was based on the total activity in the original leukocytes. Percentages of transfer were calculated by the following formulas: (i) percent of marker protein in phagosome = amount in phagolysosome fraction/total amount recovered from gradient × 100; (ii) percent of marker protein in medium = amount in medium/amount in original leukocytes × 100.

Percentage recoveries for gradients were calculated on the basis of the cell homogenates, whereas percentage recoveries for extracellular release were based on the original leukocyte suspension. Thus: (i) percent recovery for phagosomes = amount in all gradient fractions/amount in cell homogenate × 100; (ii) percent recovery for extracellular release = amount in medium + amount in cell homogenate/amount in original leukocytes × 100.

Chemical determinations and enzyme assays. Protein was measured by the method of Lowry et al. as mentioned above. The latex was measured by extraction in *p*-dioxane as described by Werb and Cohn (43). In order to analyze fractions from the gradients for latex, best results were obtained by first concentrating samples and then washing them with water to remove the sucrose. Alkaline phosphatase (28) was measured spectrophotometrically as previously described, and the neutral protease was determined using benzyloxycarbonyl-L-alanine-*p*-nitrophenyl ester as substrate (41). The enzyme 5' nucleotidase was assayed by the liberation of inorganic phosphate with 5 mM adenosine 5'-monophosphate as substrate by the method of Widnell and Unkeless (46). Where necessary, latex turbidity was cleared from the samples by filtration through membrane filters (0.8-μm pore size; Millipore Corp.).

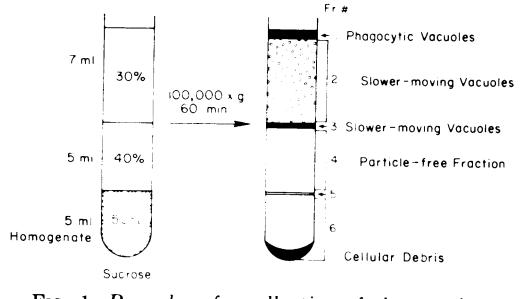

FIG. 1. *Procedure for collection of phagocytic vacuoles. Granules which failed to degranulate remained in fraction 6 with the cellular debris.*

Lactic dehydrogenase and β-glucuronidase were assayed by the methods of Bergmeyer (8) and Fishman (16), respectively. Triton (0.1%) released latent enzymes where enzymes were measured catalytically. In lactic dehydrogenase and alkaline phosphatase determinations, corrections were made for the activity contributed by the 10% AB+ normal human serum.

Immunochemical determinations of LF and MPO. The granule class markers were assayed by single, radial immunodiffusion according to Mancini (32). The purification of LF and the preparation of specific antibodies to this protein have been described before (28). MPO was purified from lysosomal granule suspensions by the procedure of Himmelhoch et al. (24) and was then used to immunize rabbits. Injections of 150 μg of MPO emulsified in complete Freund adjuvant were given subcutaneously in each of four sites, followed after 2 weeks by subcutaneous injections of the same quantity in incomplete Freund adjuvant. After 2 months, a final boost of 75 μg in incomplete Freund adjuvant was given. Both the purified MPO and the antiserum so obtained were checked by diffusion in gel for purity and specificity against standards generously donated by S. R. Himmelhoch, National Institutes of Health, Bethesda, Md.

After phagocytizing cells had been homogenized and centrifuged by the gradient procedure described, samples from the gradient fractions were concentrated by centrifugation and then extracted in 0.1% cetyltrimethyl-ammonium bromide in 0.01 M phosphate buffer, pH 8.6, before testing in the Mancini plates. Samples of the original cell suspension and the cell homogenate were also prepared. Samples of the incubation medium were concentrated twofold and were tested without cetyltrimethyl-ammonium bromide extraction. Mancini plates for MPO were washed thoroughly and then stained for peroxidase by the method of Graham and Karnovsky (19) before the precipitin rings were measured. All determinations were done in duplicate, and quantification was by standards run with each assay.

RESULTS

Gradient isolation of phagosomes. Separation of the latex bead-filled phagocytic vacuoles is shown in Fig. 1. When subjected to 100,000 × *g* centrifugation, the bead-filled vacuoles floated to the top of the gradient, with the soluble proteins and cellular debris remaining in the loading zone. Most of the latex, 40 to 50%, was found in the first fraction, but slower moving vacuoles were found throughout the 30% sucrose layer and in a second band at the 30 to 40% interface. Since the latex in the second and third fractions was very dilute, it was necessary to concentrate samples and wash them free of sucrose to obtain an accurate latex measurement by dioxane extraction. It was usually found that at least 70% of the total gradient latex was contained in the first three fractions (Table 1). The fourth fraction was particle free and served as a buffer between the vacuole fractions and the remaining cellular debris. Occasionally in fraction 5, the 40 to 50% interface, there would be a very faint band which appeared to consist of the slowest moving latex particles.

Resolution of the phagocytic vacuoles on the gradient increased the specific activity of 5′ nucleotidase to almost four times that of the cell homogenate (Table 2). The presence of the enzyme 5′ nucleotidase, which is an accepted membrane marker enzyme (43; Hawiger et al., Clin. Res. **20**:82), verified that the isolated bead fractions were membrane associated.

Degranulation into phagosomes induced by BSA-coated beads. Table 1 summarizes the chemical and immunochemical analyses of four gradient separations of phagocytic vacuoles stimulated by BSA-coated beads. In preliminary experiments the first three gradient fractions were positive for latex and 5′ nucleotidase. They contained measurable LF and MPO, but not the cytoplasmic enzyme lactic dehydrogenase; therefore, these three fractions were considered together to represent the vacuole fraction. It can be seen that the vacuoles contained 2 to 3% of the total cellular protein, 2 to 3% of the specific granule protein LF, and around 6% of the azurophil granule enzyme MPO. In addition, approximately 2% of the alkaline phosphatase activity was also found to be associated with the vacuoles. The vacuole fractions were virtually free of soluble cellular proteins, as indicated by the very low percentage of lactic dehydrogenase activity found in these fractions.

Antigen-, antibody-, and antigen-antibody complex-coated beads—complement fixation and PMN uptake. With the ultimate goal of comparing phagocytic degranulation induced by immune complexes with that induced by antigen or antibody alone, we coated beads with BSA, rabbit anti-BSA serum, and BSA-anti-BSA complexes. In no instance were beads found agglutinated. By a standard complement fixation assay, it was determined that complex-coated beads could fix as much as five times more complement than could the antibody-coated beads, whereas the BSA-coated beads fixed essentially no complement (Table 3).

It was possible that increased complement fixation could result in a greater phagocytic uptake with the complex-coated beads. Hence, it was first necessary to compare the uptakes of immune complex-coated beads with uptakes of protein-coated beads. Figure 2 shows the results of such a study performed using beads coated

TABLE 1. *Distribution of chemical components in human PMN subcellular fractions after phagocytosis of BSA-coated polystyrene latex beads*[a]

Fraction	Chemical components					
	Latex	Protein	AP[b]	LF	MPO	LDH[b]
1-3	74.9[c] ± 1.44	2.8 ± 0.2	2.3 ± 0.3	2.7 ± 0.5	6.1 ± 0.4	0.3 ± 0.2
4	5.7 ± 0.4	0.7 ± 0.2	0.8 ± 0.3	0.3 ± .03	0.2 ± 0.9	1.1 ± 0.5
5-6	19.4 ± 1.5	96.5 ± 1.4	96.9 ± 5.1	97 ± 2.6	93.7 ± 7.0	98.6 ± 5.3
Total recovery[d]	112 ± 5.7	88.2 ± 1.5	109.6 ± 5.4	83.6 ± 2.2	82 ± 5.4	84 ± 4.5

[a] Beads found in medium = half of those applied to leukocyte suspension. Amount of lactoferrin found in medium = 18% ± 1.5% of that in leukocyte suspension (i.e., amount in original leukocytes, not the amount applied to gradient). Amount of MPO found in medium = 5.3% ± 0.2% of that in leukocytes. Amount of lactic dehydrogenase found in medium = 6.3% ± 0.9 of that in leukocytes.

[b] AP, Alkaline phosphatase; LDH, lactic dehydrogenase.

[c] Percent of total recovered in gradient fraction means for four replicates ± standard error: percent in fraction = amount in fraction/total amount recovered from gradient × 100.

[d] Percent of total applied to gradient means for four replicates: percentage recovered = sum of amount in fractions/amount in cell homogenate × 100.

TABLE 2. *Comparison of the specific activities of 5' nucleotidase in human PMN cell homogenates and phagocytic vacuoles*

Determinant	Sp act[a]
Cell homogenate	5.8 ± 0.5
Phagocytic vacuoles	20.2 ± 6.6

[a] Mean ± standard error for three experiments. Specific activity expressed as units of enzymatic activity per milligram of protein.

TABLE 3. *Complement fixation by BSA, anti-BSA serum, and BSA-anti-BSA complex-coated latex beads*

Protein adsorbed to beads	50% units of complement per mg of protein
BSA	50
Anti-BSA serum	450
Complexes	2,155

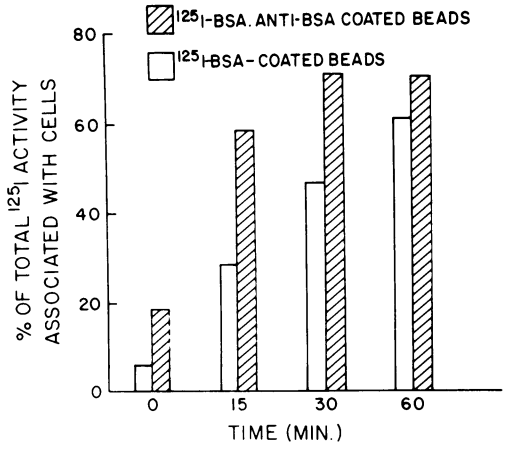

FIG. 2. *Comparison of phagocytic uptake of ^{125}I-labeled BSA and rabbit anti-BSA-^{125}I-labeled-BSA complex-coated beads by human PMN. Histograms represent the percentage of the total ^{125}I activity associated with the cells at a given time.*

with ^{125}I-labeled BSA and rabbit anti-BSA-^{125}I-labeled-BSA complexes. Cell samples were taken at 0, 15, 30, and 60 min. After thorough washing, the radioactivity remaining with the cells was measured. Cell washes were also monitored to ensure total recovery of the ^{125}I activity. It can be seen that the complex-coated beads are more rapidly ingested than are the BSA-coated beads. However, by 60 min, the total intracellular amounts are nearly equal. In this experiment, the total uptakes differed by less than 10% at 60 min. Thus, in the next experiments, incubations were performed for 60 min to allow for equalization of the latex uptakes.

Increased degranulation into phagosomes induced by immune complexes. The transfer of granule proteins into phagosomes using antigen-, antibody-, and immune complex-coated beads is compared in Fig. 3. For all proteins and enzymes assayed the antigen-antibody complexes appeared to induce more degranulation into the phagosomes that did antigen or antibody alone. The antibody-coated beads also seemed to be a slightly better stimulus than was antigen alone. The immune complexes induced a slight but significant increase in the LF associated with the vacuoles, but they simultaneously induced more than a twofold increase in the MPO. Significantly, the immune complexes

stimulated the greatest degranulation of the neutral protease, an enzyme also localized in the azurophil granule. Because of a known inhibitor for the protease within PMN (26), this enzyme was difficult to quantify and, therefore, could not be taken as a reliable one for measuring azurophil degranulation. The vacuole fractions, separated from the cellular debris, cytosol and the inhibitor, could have been displaying disproportionately high activities for this enzyme. It is apparent, nevertheless, that immune complexes stimulate the greatest degree of degranulation from both granule classes, but of the two classes the azurophils contribute significantly more to the phagosomes than do the specifics.

Increased degranulation into extracellular medium induced by immune complexes. In the same series of experiments, when we followed the release of granule proteins into the media, antigen-antibody complexes also induced more degranulation than did antigen or antibody alone, but now the greatest release was of the specific granule protein LF (Fig. 4). The immune complexes induced the release of up to 50% of the LF into the media. In the extracellular medium the proteolytic enzyme may be underestimated because of serum inhibitors, but the MPO measured by immunodiffusion should be an accurate measure of azurophil degranulation. MPO, in contrast to the LF, was released in amounts only slightly higher than that of lactic dehydrogenase, which is an indicator of leakage due to cell damage. The amount of alkaline phosphatase actively released into the media was comparable to that released of MPO and the protease, but was significantly lower than that of LF. Equal proportions of alkaline phosphatase left the cells regardless of the kind of coating on the particles.

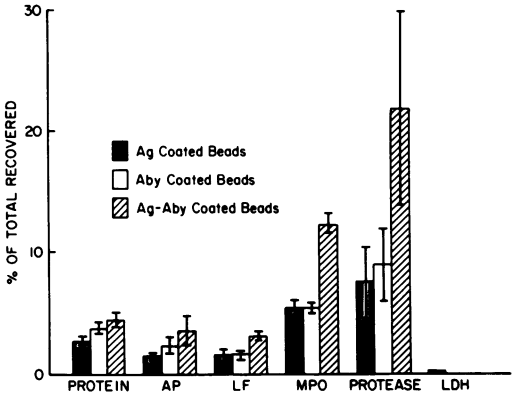

FIG. 3. *Transfer of PMN granule components into phagocytic vacuoles stimulated by antigen-, antibody-, and antigen-antibody complex-coated latex beads. Histograms show the means for three experiments with standard error indicated by brackets. Percentage recoveries for antigen, antibody, and antigen-antibody complex gradients in respective order were as follows. Protein: 95.5 ± 1.0, 94.0 ± 2.5, 98.5 ± 1.8; alkaline phosphatase (AP): 109.0 ± 15.2, 89.3 ± 0.4, 90.7 ± 3.9; lactoferrin (LF): 91.7 ± 4.0, 95.7 ± 2.0, 86.3 ± 6.8; myeloperoxidase (MPO): 87.6 ± 9.1, 86.7 ± 6.6, 80.3 ± 7.7; protease: 72.3 ± 7.7, 78.0 ± 6.0, 105.3 ± 15.9; lactic dehydrogenase (LDH): 96.5 ± 5.2, 90.8 ± 1.2, 88.3 ± 3.5.*

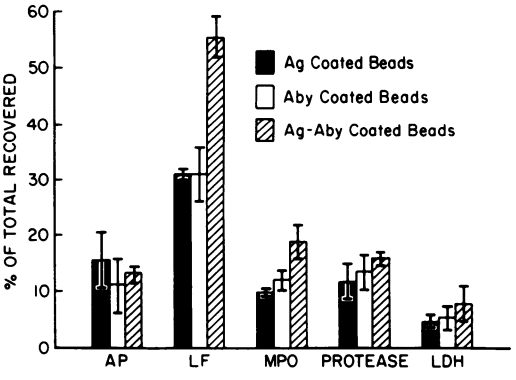

FIG. 4. *Transfer of PMN components into extracellular medium stimulated by antigen-, antibody-, and antigen-antibody complex-coated latex beads. Histograms show the means with standard error for three experiments. Percentage recoveries for enzyme releases induced by antigen, antibody, and complex were, in respective order: alkaline phosphatase (AP): 70.9 ± 4.6, 78.1 ± 14.9, 77.3 ± 10.4; lactoferrin (LF): 94.2 ± 8.4, 84.8 ± 14.4, 98.9 ± 0.8; myeloperoxidase (MPO): 99.3 ± 9.5, 89.8 ± 15.0, 100.1 ± 10.1; protease: 89.8 ± 1.8, 85.9 ± 16.7, 57.9 ± 3.5; lactic dehydrogenase (LDH): 108.5 ± 7.0, 91.2 ± 8.3, 99.6 ± 12.5.*

Time studies of degranulation. The time course of degranulation was studied in a series of experiments shown in Fig. 5. Degranulation stimulated by rabbit IgG complexes in a 0.3 ratio was compared with that stimulated by BSA-coated beads. Samples were taken at 15, 30, and 60 min. It can be seen that the prepared immune complexes induced more overall degranulation and at a faster rate than did the antigen-coated beads. In either case, the specific granule marker LF was found in the medium much sooner and to a greater extent than was the azurophil marker, MPO. Conversely, looking at the phagosomes, MPO appeared to enter the vacuoles at a faster rate and to a greater extent than did LF. The release of β-glucuronidase, an enzyme also included in the azurophil class, closely paralleled that of MPO.

During the transfer of alkaline phosphatase, its extracellular release was slightly greater

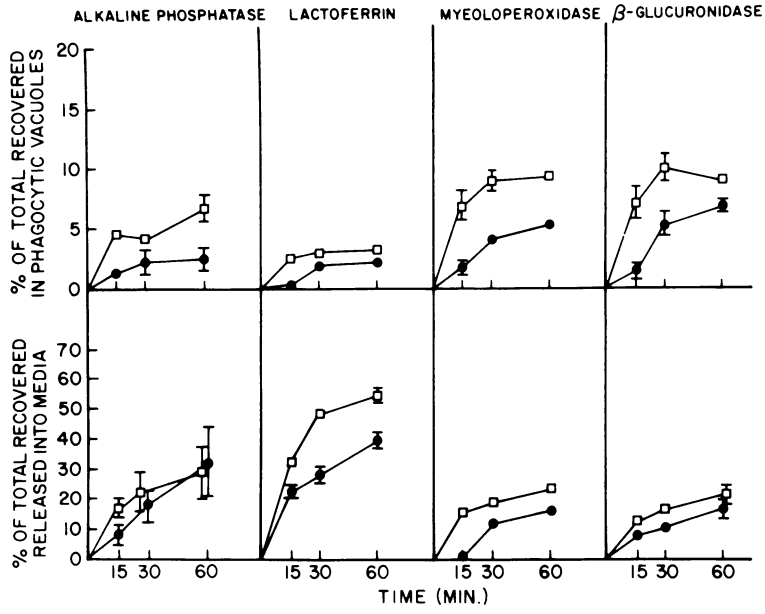

FIG. 5. *Transfer of PMN components into phagocytic vacuoles and extracellular medium as a function of time. Degranulation stimulated by rabbit anti-BSA IgG-BSA complex-coated beads (□) was compared with that stimulated by BSA-coated beads (●). Results are expressed as the means of two duplicate experiments, with the range indicated by brackets. Each point represents the percentage of activity or protein in vacuole fraction or medium relative to the total activity or protein at a given time. In all cases the percentages recovered were greater than 80% and usually were at least 90%. Where brackets are not shown, the range was smaller than the symbol for that point.*

than that of MPO and β-glucuronidase, but was significantly lower than that of LF. Whereas immune complexes significantly increased the release of LF, such complexes induced no greater release of alkaline phosphatase than did antigen alone. On the average, 6% of the total cellular lactic dehydrogenase was released into the medium, and the release did not increase with time, which suggests that the granule enzyme release did not result from or cause cell damage.

DISCUSSION

The use of immunochemical assays for LF and MPO, respectively, as markers for specific and azurophil granules affords major advantages. Previous studies have most often employed release of various granule enzymes, including β-glucuronidase (20, 21, 47), alkaline phosphatase (20, 21, 33), lysozyme (33, 39, 47), and catalytically measured peroxidase (22), as criteria for degranulation, but until now no unambiguous markers have been available for the two recognized granule classes. β-glucuronidase is localized in the azurophil granule, but a substantial amount of its activity within PMN may be from other subcellular sites (2, 7). Alkaline phosphatase has been used as a marker for the specific granule in rabbit PMN on the basis of its histochemical localization (5). Alkaline phosphatase is not a component of specific granules of human PMN, however. As we have shown, this enzyme is separable from specific granules by sucrose density centrifugation (28, 35). This finding has been confirmed (1, 36; B. Gusus, U. Bretz, and M. Baggiolini, Experentia **29**:772; B. C. West, N. A. Gelb, and H. R. Kimball, Fed. Proc. **31**:253). Lysozyme is unsuitable as a specific marker for either granule class, since it is included in both classes (2, 28). As we searched for azurophil granule markers, we could not distinguish with spectrophotometric assay between MPO and other peroxidases, such as the one found in the eosinophil. However, an immunochemical distinction between neutrophilic and eosinophilic peroxidases can be made (31, 34, 36). Moreover, LF, measured immunochemically, is a constituent of human specific granules (28). Thus, immunochemical assays for both LF and MPO make it possible to follow degranulation with markers specific not only for the neutrophil, but also for its two granule classes.

Apparently, the surface characteristics of in-

gested particles can influence the degree of degranulation. In preliminary studies, we were unable to detect any significant degranulation into phagosomes stimulated by uncoated latex beads. Hawiger et al. were also unable to detect any granule enzyme activity in their bead phagosomes (Clin. Res. **20**:82). By using beads coated with BSA, however, we induced measurable protein release into the phagosomes from both PMN granule classes. The BSA conceivably could have done this by binding a trace of anti-BSA normally present in the human serum used in the phagocytosis experiments, although this has not been established. The important point is that the beads coated with immune complexes induced even greater release of granule constituents than beads coated only with BSA. That is, with an uptake of nearly equal amounts of latex beads, twice as much granule protein was released into both vacuoles and extracellular medium when the beads were coated with immune complexes as when they were coated with antigen or antibody alone. The increase in protein adsorbed to the beads was not sufficient to account for the observed increase in degranulation, as a less than twofold increase in protein produced a greater than twofold degranulation.

The properties of immune complexes that lead to greater degranulation may be related to their chemotactic potential and their ability to fix complement. In the uptake studies it was shown that immune complex-coated beads were ingested more rapidly than antigen-coated beads, although the uptakes equalized after 1 h. It could be that the greater release induced by immune complexes is simply due to an earlier onset of degranulation. We have not tried to evaluate the role of complement in our system, except to insure that it was always present. As there is ample evidence for complement-increased chemotaxis and phagocytosis (17), the greater complement fixation of immune complex-coated beads could certainly explain their more rapid uptake. Recently Goldstein and Weissman have reported that complement activation by the alternate pathway can increase extracellular granule enzyme release in cytochalasin B-treated PMN (18). Thus, complement may be contributing to the greater degranulation induced by immune complexes in our system.

During degranulation, there was a differential release of granule class proteins, with more of the specifics being released into the extracellular medium whereas more of the azurophils remained with the phagocytic vacuoles. Other workers have reported extracellular release of granule enzymes during phagocytosis (20, 21, 33, 39, 47); however, selective release of a particular granule class has not been heretofore emphasized. In 1964, Ohta did report that large amounts of lysozyme from horse PMN were released into extracellular medium during phagocytosis of bacteria, whereas other enzymes, notably alkaline phosphatase, were not (33). Taichman et al. have subsequently demonstrated a greater release of lysozyme than of other granule constituents in response to immune complexes (39). These findings are consistent with ours as 50% of PMN lysozyme is associated with LF in the specific granules (28).

An explanation for this phenomenon of greater specific granule extracellular release is not readily available. There could be a tendency for MPO to remain bound to membranes after azurophil degranulation. This could explain why it was not released into the medium in large quantities, but other azurophil enzymes, neutral protease and β-glucuronidase, were not released into the medium in large quantities either. Bainton et al. have suggested that in rabbit PMN there is a sequence of degranulation, the specifics first emptying their contents into phagocytic vacuoles (3). If this is so, then it follows that degranulation of the specific granules could be occurring before the latex beads have been completely enclosed by the cell; thus, large amounts of this protein could be released into the medium. If azurophil degranulation occurs later, as vacuoles are being closed, then lesser amounts of these proteins would escape.

We observed a transfer of alkaline phosphatase into the medium. In Henson's model of "frustrated" phagocytosis, immune complexes immobilized on membranes stimulated cellular release of alkaline phosphatase, but it remained bound to the outside of the cells (23). That we did observe some extracellular release may simply be due to the difference in the systems —"consummated" versus "frustrated" phagocytosis or rabbit versus human PMN. Granule release of MPO into phagosomes and extracellular medium was paralleled by two other known azurophil enzymes, β-glucuronidase and neutral protease, but the release of alkaline phosphatase into the medium was not parallel to that of the known specific granule protein LF. Equal quantities of ingested latex induced essentially equal alkaline phosphatase release, regardless of the protein coating on the beads, whereas for LF immune complexes induced significantly greater extracellular release than did either antigen or antibody alone. Since

there is substantial evidence (1, 28, 35, 36; Gusus et al., Experentia **29**:772; West et al., Fed. Proc. **31**:253) that alkaline phosphatase is a component of cell membranes and not a component of any granule, it seems quite logical that this enzyme would not necessarily be released proportionately to a protein, i.e., LF, that is truly included within the granule matrix.

Comparing the release of LF and MPO within our system, two conclusions can be drawn. First, that immune complexes can induce more overall degranulation than can antigen or antibody alone, and, secondly, that degranulation of the specific and azurophil granule classes of human PMN occur as independent phenomena. Finally, we must emphasize that for specific granules of human PMN, LF, to date, appears to be the only unambiguous marker. Lysozyme is an ambiguous marker because it is distributed equally between specific and azurophil granules. Alkaline phosphatase, upon which others have relied in their identification of specific granules (14, 38, 45), is not acceptable as a marker because in human PMN it is not associated with specific granules (1, 28, 35, 36; West et al., Fed. Proc. **31**:253).

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LITERATURE CITED

1. Baggiolini, M., U. Bretz, and B. Gusus. 1974. Biochemical characterization of azurophil and specific granules from human and rabbit polymorphonuclear leukocytes. Schweiz. Med. Wochenschr. **104**:129-132.
2. Baggiolini, M., J. G. Hirsch, and C. de Duve. 1969. Resolution of granules from rabbit heterophil leukocytes into distinct populations by zonal sedimentation. J. Cell Biol. **40**:529-541.
3. Bainton, D. F. 1973. Sequential degranulation of the two types of polymorphonuclear leukocyte granules during phagocytosis of microorganisms. J. Cell Biol. **58**:249-264.
4. Bainton, D. F., and N. G. Farquhar. 1966. Origin of granules in polymorphonuclear leukocytes. Two types derived from opposite faces of the Golgi complex in developing granulocytes. J. Cell Biol. **28**:277-301.
5. Bainton, D. F., and M. G. Farquhar. 1968. Differences in enzyme content of azurophil and specific granules of polymorphonuclear leukocytes. II. Cytochemistry and electron microscopy of bone marrow cells. J. Cell Biol. **39**:299-317.
6. Bainton, D. F., J. L. Ullyot, and M. G. Farquhar. 1971. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. Origin and content of azurophil and specific granules. J. Exp. Med. **134**:907-934.
7. Beaufay, H. 1972. Methods for the isolation of lysosomes, p. 1. 1-45. *In* J. T. Dingle (ed.), Lysosomes, a laboratory handbook. North-Holland Publishing Co., Amsterdam.
8. Bergmeyer, H.-U., 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.
9. Campbell, D. H., J. S. Garvey, N. E. Cremer, and D. H. Sussdorf. 1963. Methods in immunology, p. 172. W. A. Benjamin, Inc., New York.
10. Cline, M. J. 1970. Leukocyte function in inflammation: the ingestion, killing, and digestion of microorganisms. Ser. Haematol. **III**:3-16.
11. Cochrane, C. G. 1968. Immunologic tissue injury mediated by neutrophilic leukocytes. Advan. Immunol. **9**:97-162.
12. Cuatrecasas, P., and C. B. Anfinsen. 1971. Affinity chromatography. Annu. Rev. Biochem. **40**:259-278.
13. Dresser, D. W., and H. H. Wortis. 1967. Localized haemolysis in gel, p. 1068. *In* D. M. Weir (ed.), Handbook of experimental immunology. F. A. Davis Co., Philadelphia.
14. Estensen, R. D., J. G. White, and B. Holmes. 1974. Specific degranulation of human polymorphonuclear leukocytes. Nature (London) **248**:347-348.
15. Fallon, H. J., E. Frei, J. D. Davidson, J. S. Trier, and D. Burk. 1962. Leukocyte preparations from human blood: evaluation of their morphologic and metabolic state. J. Lab. Clin. Med. **59**:779-791.
16. Fishman, W. H. 1963. β-glucuronidase, p. 869-874. *In* H.-U. Bergmeyer (ed.), Methods of enzymatic analysis. Academic Press Inc., New York.
17. Gigli, I., and R. A. Nelson, Jr. 1968. Complement dependent immune phagocytosis. I. Requirements for C'1, C'4, C'2, C'3. Exp. Cell Res. **51**:45-67.
18. Goldstein, I. M., M. Brai, A. G. Osler, and G. Weissmann. 1973. Lysosomal enzyme release from human leukocytes: mediation by the alternate pathway of complement activation. J. Immunol. **111**:33-37.
19. Graham, R. C., Jr., and M. J. Karnovsky. 1966. The early stages of adsorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. Cytochemistry **14**:291.
20. Hawkins, D., and S. Peeters. 1971. The response of polymorphonuclear leukocytes to immune complexes *in vitro*. Lab. Invest. **24**:483-491.
21. Henson, P. M. 1971. Interaction of cells with immune complexes: adherence, release of constituents, and tissue injury. J. Exp. Med. **134**:114s-135s.
22. Henson, P. M. 1971. The immunologic release of constituents from neutrophil leukocytes. I. The role of antibody and complement on nonphagocytosable surfaces or phagocytosable particles. J. Immunol. **107**:1535-1546.
23. Henson, P. M. 1971. The immunologic release of constituents from neutrophil leukocytes. II. The mechanisms of release during phagocytosis, and adherence to nonphagocytosable surfaces. J. Immunol. **107**:1547-1557.
24. 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**:914-921.
25. Janoff, A. 1972. Neutrophil proteases in inflammation. Annu. Rev. Med. **23**:177-190.
26. Janoff, A., and J. Blondin. 1971. Further studies on an esterase inhibitor in human leukocyte cytosol. Lab. Invest. **25**:565-571.
27. Klebanoff, S. J. 1970. Myeloperoxidase: contribution to the microbicidal activity of intact leukocytes. Science **169**:1095-1097.
28. Leffell, M. S., and J. K. Spitznagel. 1972. Association of lactoferrin with lysozyme in granules of human poly-

morphonuclear leukocytes. Infect. Immunity **6**:761-765.
29. 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.
30. McFarlane, A. S. 1958. Efficient trace-labelling of proteins with iodine. Nature (London) **182**:53.
31. Mage, M. G., W. H. Evans, S. R. Himmelhoch, and L. McHugh. 1971. Immunological identification and quantification of guinea pig heterophil and eosinophil peroxidases. J. Reticuloendothel. Soc. **9**:201-208.
32. Mancini, G., A. O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry **2**:235-254.
33. Ohta, H. 1964. A biochemical study on the neutrophilic granules isolated in a pure state from leukocyte homogenate. Acta Haematol. Japan. **27**:555.
34. Salmon, S. E., M. J. Cline, J. Schultz, and R. I. Lehrer. 1970. Myeloperoxidase deficiency. Immunologic study of a genetic leukocyte defect. New Engl. J. Med. **282**:250-253.
35. Spitznagel, J. K. 1971. Sorting out lysosomes and other cytoplasmic granules from polymorphs of rabbits and humans: a search for antibacterial factors, p. 83-106. *In* R. C. Williams and H. H. Fudenberg (ed.), The Kroc Foundation symposium on phagocytic mechanisms in health and disease. Intercontinental Medical Book Corp., New York.
36. Spitznagel, J. K., F. G. Dalldorf, M. S. Leffell, J. D. Folds, I. R. H. Welsh, M. H. Cooney, and L. A. Martin. 1974. Character of azurophil and specific granules purified from human polymorphonuclear leukocytes. Lab. Invest. **30**:774-785.
37. Stossel, T. P., T. D. Pollard, R. J. Mason, and M. Vaughan. 1971. Isolation and properties of phagocytic vesicles from polymorphonuclear leukocytes. J. Clin. Invest. **50**:1745-1757.
38. Strauss, R. G., K. E. Bove, J. F. Jones, A. M. Mauer, and V. A. Fulginitti. 1974. An anomaly of neutrophil morphology with impaired function. N. Engl. J. Med. **290**:478-484.
39. 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 Appl. Immunol. **43**:182-195.
40. Van Oss, C. J., and J. M. Singer. 1966. The binding of immune globulins and other proteins by polystyrene latex particles. J. Reticuloendothel. Soc. **3**:29-40.
41. Visser, L., and E. R. Blout. 1972. The use of *p*-nitrophenyl *N-tert*-butyloxycarbonyl-L-alaninate as substrate for elastase. Biochim. Biophys. Acta **268**:257-260.
42. Weissmann, G., R. B. Zurier, P. J. Spieler, and I. M. Goldstein. 1971. Mechanisms of lysosomal enzyme release from leukocytes exposed to immune complexes and other particles. J. Exp. Med. **134**:149s-165s.
43. Werb, Z., and Z. A. Cohn. 1972. Plasma membrane synthesis in the macrophage following phagocytosis of polystyrene latex particles. J. Biol. Chem. **247**:2439-2446.
44. Wetzel, M. G., and E. D. Korn. 1969. Phagocytosis of latex beads by *Acanthamoeba castellanii* (Neff). III. Isolation of the phagocytic vesicles and their membranes. J. Cell Biol. **43**:90-104.
45. White, J. G., and R. D. Estensen. 1974. Selective labilization of specific granules in polymorphonuclear leukocytes by phorbol myristate acetate. Amer. J. Pathol. **75**:45-54.
46. Widnell, C. C., and J. C. Unkeless. 1968. Partial purification of a lip-protein with 5'-nucleotidase activity from membranes of rat liver cells. Proc. Nat. Acad. Sci. U.S.A. **61**:1050-1057.
47. Wright, D. G., and S. E. Malawista. 1972. The mobilization and extracellular release of granular enzymes from human leukocytes during phagocytosis. J. Cell Biol. **53**:788-797.
48. Zeya, H. I., and J. K. Spitznagel. 1968. Arginine-rich proteins of polymorphonuclear leukocyte lysosomes. Antimicrobial specificity and biochemical heterogeneity. J. Exp. Med. **127**:927-941.