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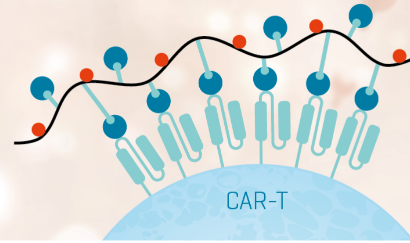

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THE SEQUENTIAL RELEASE OF GRANULE CONSTITUENTS FROM HUMAN NEUTROPHILS

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The degranulation response of human neutrophils to the calcium ionophore A23187, serum opsonized zymosan (ZC), aggregated γ-globulin (AγG), C5a, formyl-methionyl-leucyl-phenylalanine (FMLP), and PMA has been studied as a reaction time course in order to compare the release kinetics of the separate granule types. Cell suspensions were treated with submaximal doses of stimuli for various time intervals, and the isolated supernatants were assayed for granule constituents. Lactoferrin (LF), a unique specific (secondary) granule protein, was measured by radioimmune assay, and the azurophil (primary) granule components, myeloperoxidase (MPO) and β-glucuronidase (β-glu), by enzymatic activity. A sequential pattern of first LF release followed by MPO and β-glu was demonstrated with each of the stimuli examined, with or without cytochalasin B pretreatment.

These kinetic studies demonstrate that the extracellular release of the specific and azurophil granules occur sequentially in human neutrophils with both soluble and particulate stimuli. These findings support the concept that the two granule types are subject to separate controlling factors.

As active participants in host defense, neutrophils (polymorphonuclear leukocytes) can discharge the contents of their storage granules both into phagosomes and to the external environment. This external release occurs 1) during the process of phagocytosis (1-6), 2) when cells react with soluble stimuli bound to surfaces too extensive for engulfment (7-9), or 3) in response to the same soluble stimuli in the presence of the fungal metabolite, cytochalasin B (CB)[1] (10-12). This granule discharge is triggered at the neutrophil plasma membrane (9, 13) resulting in granule mobilization and fusion with the external membrane (1), but the intracellular control mechanisms for the process are as yet undefined.

Rabbit neutrophils contain two predominant granule classes as distinguished by morphogenesis and cytochemistry (14, 15) and by biochemical analysis after various cell fractionation procedures (16-18). The azurophil (primary) granules represent the true lysosomes of the cell, containing the acid hydrolases such as β-glucuronidase (β-glu), β-galactosidase, and acid cathepsins. They also contain, however, neutral proteases, myeloperoxidase (MPO), and a portion of the cellular lysozyme (lys) (19). The specific (secondary) granules are formed late during neutrophil differentiation within the bone marrow and contain lys, collagenase, alkaline phosphatase (16, 17), lactoferrin (LF) (18, 20), and a vitamin B_{12} binding protein (21). As has been suggested from observations of sequential degranulation (22) and release (9), the discharge of these two granule types may be under differential control. During bacterial phagocytosis, Bainton (22) demonstrated fusion of the specific granules with the phagosome before azurophil granule fusion. Also, rabbit neutrophils stimulated with surface-bound aggregated γ-globulin (23) released specific granule contents before those of the azurophil granules. Jensen and Bainton (23a) postulated that this degranulation sequence facilitates optimal conditions for coordinated biochemical activities of the various granule components within the phagosome for "killing" and subsequent digestion.

Human neutrophils have not been as well characterized. Although there are at least two granule classes as distinguished by peroxidase cytochemistry (14, 15), cell fractionation studies describe up to four granule types as separated by sucrose gradient modal densities (21, 24, 25-27). Human neutrophils may contain subclasses of granules with overlapping contents and different degranulation properties. Although a sequential degranulation process has not been definitively shown for human neutrophils, the release of granule components also appears to be subject to differential control. A largely selective release of specific granules can be induced by phorbol myristate acetate (PMA) (28-30), concanavalin A (Con A) (31, 32), and by the calcium ionophore, A23187, or by calcium alone (33-36). Specific granules, apparently more numerous than the azurophil granules (16, 21), are released more readily and to a greater degree (5). It has also been suggested that the specific granules may actually be secretory granules whose contents function extracellularly (6, 37).

The present study was undertaken to examine the extracellular release kinetics of human neutrophil granule constituents with a view toward determining whether sequential discharge of separate granule types occurs in this species. These parameters are addressed by examining the release of LF, MPO, β-glu, and lys from neutrophils in response to various soluble and particulate stimuli.

MATERIALS AND METHODS

Preparation of neutrophils. Blood from normal human donors was drawn by venipuncture into 1/10 volume of 3.8% citrate

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[1] Abbreviations used in this text: Hanks' BSA, Hanks' balanced salt solution with 0.25% bovine serum albumin; DMSO, dimethyl sulfoxide; CB, cytochalasin B; ZC, serum opsonized zymosan; FMLP, formyl methionyl-leucyl-phenylalanine; PMA, phorbol myristate acetate; SaγG, soluble aggregated γ-globulin; BDB, bis-diazotized benzedine; LF, lactoferrin; Rab anti-LF, rabbit anti-lactoferrin; MPO, myeloperoxidase; lys, lysozyme; β-glu, β-glucuronidase; LDH, lactic dehydrogenase; EDTA, ethylenediaminetetraacetic acid; RIA, radioimmune assay.

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or into acid citrate dextrose in a 50-ml conical plastic tube (Falcon Plastics, Oxnard, Calif.), as described by Aster and Jandl (38). These were centrifuged at 300 × G for 20 min at room temperature, and the platelet-rich plasma was aspirated. The platelets were removed by centrifugation at 2500 × G for 15 min to provide platelet-poor plasma. To the remaining blood was added 5 ml of 6% dextran T-500 (Pharmacia Fine Chemicals, Piscataway, N. J.) in saline and additional saline to bring the volume to 50 ml. After a 30-min sedimentation, cells in the supernatant were pelleted at 275 × G for 10 min and resuspended in 8 ml platelet-poor plasma diluted 1:4 in saline. This was layered on 3 ml Ficoll-Hypaque (Pharmacia, 1.077 g/ml) in 16 × 125 plastic tubes (Falcon). The cells, which pelleted at 750 × G, 25 min at 18°C, were removed, resuspended in erythrocyte lysing solution consisting of 8.29 g/liter NH_4Cl, 0.037 g/liter Na_2 EDTA (see Abbreviations) and 1 g/liter $KHCO_3$ (39), centrifuged at 275 × G for 5 min, washed twice in Hanks' balanced salt solution with 0.25% bovine serum albumin (Hanks' BSA), resuspended to 8×10^6/ml, and kept at 4°C until used (within 1 hr). The cells represented more than 98% granulocytes (usually 97% neutrophils, 3% eosinophils) and were routinely more than 98% viable by trypan blue exclusion and by a lack of release of the cytoplasmic enzyme lactic dehydrogenase (LDH).

Preparation of stimuli and reagents. For incubations of cells with soluble reagents, CB from Aldrich Chemical Co., Milwaukee, Wis., was dissolved in dimethyl sulfoxide (DMSO) to either 5 mg/ml or 0.5 mg/ml. One microliter was added to 1 ml final volume reaction mixtures (5 μg/ml and 0.5 μg/ml, final concentrations). This concentration of solvent was previously determined to have no injurious effect on cells or their secretion properties. The complement (C) fragment C5a was purified to homogeneity as previously described and graciously provided by Dr. Robert Webster (Webster, R. O. 1980. The biological effects of the human complement fragments C_5a and C_5a des arg on neutrophil function. Immunopharmacology. In press.). The tripeptide formyl-methionyl-leucyl-phenylalanine (FMLP) (Vega-Fox Biochemicals, Tucson, Ariz.) was dissolved in Hanks' at stock concentrations of 10^{-4} M and diluted before use in Hanks' BSA. Phorbol myristate acetate (PMA) was purchased from Consolidated Midland Corp., Katona, N. Y., dissolved and diluted to appropriate concentrations in 1 μl of DMSO for a 1-ml reaction volume. Opsonized zymosan (ZC) was boiled, washed, and incubated with normal human serum for 30 min at 37°C to fix C and again thoroughly washed with saline. Soluble aggregated γ-globulins (SaγG): normal human γ-globulin, Cohn fraction II (HG-II, Sigma Chemical Co., St. Louis, Mo.) was aggregated with bis-diazotized benzedine (BDB) by a modified method of Ishizaka *et al.* (40, 41). A mixture of 5 mg/ml HG-II in borate buffer, pH 8.5, with 20 mg BDB yielded soluble aggregates (9). Aliquots of each preparation were centrifuged before use to pellet any insoluble aggregates and assayed to standardize their range of effectiveness as stimuli for neutrophils. Calcium ionophore A23187 was supplied by Dr. Robert L. Hamill of Lilly Research Laboratories, Indianapolis, Indiana, and prepared for use in DMSO at concentrations requiring only 1 μl of solvent per ml of reaction mixture.

Release experiments. Duplicate samples of neutrophils were incubated in 1.5 ml Eppendorf (Brinkman Instruments, Westbury, N. Y.) centrifuge tubes at a final concentration of 4×10^6 cells in 1 ml. CB was used at concentrations previously shown to give maximal effect: 5 μg/ml for the soluble stimuli (C5a, FMLP, SaγG), and 0.5 μg/ml for insoluble stimuli (ZC) (Henson and Henson, unpublished observations). Cell suspensions were treated at 37°C with or without CB for 5 min before addition of stimulus. Incubations were performed at 37°C and stopped by immediate centrifugation in a Brinkman Eppendorf centrifuge. At 15,000 × G the cells were pelleted within 2 sec. Supernatants were then set directly on ice, and aliquots were assayed for the release of neutrophil granule constituents. Maximum elapsed time required for centrifugation and cooling for any time point was routinely less than 30 sec. Processing in this way, by using rapid centrifugation, caused no granule release from unstimulated cells, and the earliest time points with stimuli consistently showed little or no release. Cell death was monitored by LDH release, which did not exceed 2.5% with any stimuli used.

Assays for neutrophil granule constituents

Enzyme assays. Duplicate cell supernatant aliquots were assayed for the granule marker enzymes lys, MPO, β-glu, and cytoplasmic enzyme LDH by using methods described previously (2, 42). The amount of granule release was calculated as a percentage of the total enzyme available from neutrophil suspensions after lysis with 0.1% Triton X-100. For a direct comparison of the kinetic data from separate enzyme assays, the data from each assay was normalized to the same 100% end point. The maximal release achieved with a given stimulus for each granule marker was used as the end point, and each time point was expressed as a function thereof. A point-for-point analysis of variance was performed to critically assess the statistical differences between the kinetic curves for each granule marker. Granule constituents were not appreciably absorbed by any of the stimuli within the incubation times used.

LF radioimmune assay (RIA). LF was assayed in experimental supernatants or cell lysates by its capacity to inhibit ^{125}I-labeled antigen binding to a specific primary antisera. Purified LF from human milk (Calbiochem, La Jolla, Calif.) showed a single band on gel electrophoresis and a reaction of identity with human neutrophil supernatants or lysates in Ouchterlony analysis by using monospecific rabbit anti-LF (Rab anti-LF, Bio Rad Laboratories, Richmond, Calif.). This LF was labeled with ^{125}I (ICN, Cleveland, Ohio) either by a lactoperoxidase method (43) or with chloramine T (44). Serial dilutions of unlabeled LF were used to generate a standard curve of binding inhibition with a nonsaturating dilution of Rab anti-LF and a known amount of ^{125}I-LF. The unlabeled LF standard was prepared at 1 mg/ml in 0.1 M phosphate buffer, pH 8.0, with 0.5 M NaCl to prevent protein aggregation (5, 45). This salt concentration was shown to have no effect on primary antigen binding after dilution in the incubation media. Fifty-microliter aliquots of either the LF standards or of the experimental supernatants were incubated for 30 min at 37°C with 100 μl 5% bovine serum albumin, 50 μl of Rab anti-LF diluted 1/100 and 100 ng ^{125}I-LF, all in 0.1 M phosphate buffer. A 50-μl aliquot of monospecific goat anti-rabbit IgG, previously shown to precipitate all the rabbit antibody, was added and incubated overnight at 4°C. Tubes were subsequently centrifuged 10 min in a Brinkman Eppendorf centrifuge, and precipitates were washed twice with cold 0.1 M phosphate buffer, pH 8.0. Pellets were counted in a Beckman Gamma 7000 counter. Radiolabeled antigen binding was expressed as a percentage of the total counts precipitated by cold 10% trichloroacetic acid in control tubes minus counts precipitated by nonimmune rabbit serum control samples. LF released from stimulated neutrophils was expressed as a percentage of the total LF in cells as assessed from 0.1% Triton X-100 lysates in Hanks' BSA with additional 0.5 M NaCl to maintain LF solubility. The range of sensitivity of this assay was between 0.05 and 25.0 ng/ml LF. Suspensions

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of 4 × 10^6 neutrophils/ml contained between 15 and 20 μg of LF, which agrees well with the amounts previously reported (20, 45).

For each stimulus, experiments were performed at least three times in duplicate by using cell preparations from different donors.

RESULTS

In preliminary experiments, optimal incubation times were determined to observe initial and maximal granule release with each of the various stimuli. The soluble chemotactic stimuli C5a, FMLP and SaγG induced maximal degranulation of CB-treated cells within 2 min, whereas PMA, A23187, and the particulate stimuli required incubation times up to 1 hr for maximal effect. Concentrations of stimuli were selected to yield approximately a 20 to 40% release of the total lysozyme in Triton X-100 lysate. MPO and β-glu were selected as markers for the azurophil granules, and LF for the specific granules. Lysozyme was also used as a specific granule marker, recognizing that a portion is also contained in the azurophil granules. Experiments were performed both with and without CB to determine the effect of this drug on the kinetics and order of granule discharge.

The results are presented here in two ways: a) as the percent of the total available marker that was released, i.e., the amount of granule constituent released as a percentage of the total available after 0.1% Triton X-100 lysis, and b) as a percentage of the maximal release achieved at this stimulus concentration for each marker. In this latter presentation the individual curves are normalized as described in the *Methods* section. This data presentation compensates for variations between individual donors, between separate granule markers, and between results obtained with various stimuli.

Kinetics of ionophore-induced release. Initial experiments were performed to examine the kinetics of release induced by the calcium ionophore A23187. This agent is postulated to bypass surface receptors and to induce degranulation by enhanced intracellular calcium mobilization. Previous studies have reported a dissociation of specific and azurophil granule release by modulation of either ionophore or calcium ion concentration (33, 36).

The kinetics of granule release with the use of 2.5 × 10^{-6} M ionophore in the presence of 1.25 mM calcium is shown in Figure 1. Lysozyme was released earlier and to a greater extent than MPO in the absence of CB. The effect of CB pretreatment was to enhance the extent of release for both markers (Fig. 1*a*) without affecting the relative rates (Fig. 1*b*). The difference between the rates of lysozyme and MPO release was similar with or without CB, and in both cases, the release was sequential.

Degranulation kinetics with particulate stimuli. To determine whether a sequential release process could be observed with a physiologic stimulus, the kinetics of granule discharge with ZC were examined. ZC is known to induce mobilization and degranulation of both specific and azurophil granules from normal cells during the process of phagocytosis. In Figure 2, ZC induced maximal LF release within 10 to 20 min. MPO and β-glu release was delayed and was slower in reaching maximum. Cell preparations from the same donor, when pretreated with 0.5 μg/ml CB, responded to ZC with enhanced overall constituent release (Fig. 3*a*). The relative release order of the two granule types, however, was not altered (Fig. 3*b*).

Degranulation kinetics with soluble stimuli. Purified C5a, FMLP, and SaγG stimulated maximal degranulation of CB-

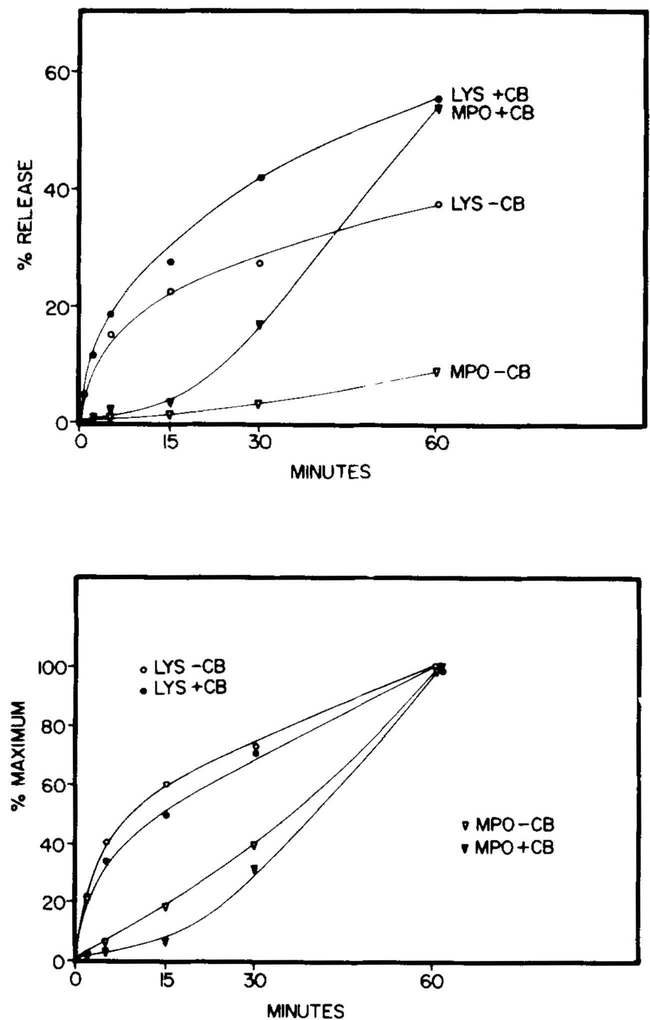

Figure 1. Kinetics of neutrophil granule release induced by 2.5 × 10^{-6} M A23187 with or without 5 μg/ml CB. *A*, enzyme release as percent of total available in detergent lysate; *B*, as a percent of the maximal release. Note from *Materials and Methods* that the zero time point incorporates 30 sec of processing and centrifugation time.

treated neutrophils within 2 min in these and previous studies (41, 42). These stimuli also allowed an examination of the granule release kinetics without internalization during phagocytosis. The initial rates of granule discharge were examined by assaying cell supernatants at the earliest possible time points. With each of these stimuli, CB at 5 μg/ml was used to induce high percentages of granule constituent release. Figure 4 demonstrates the response of CB-treated neutrophils to SaγG. A substantial portion of the LF was released within 5 sec. MPO and β-glu release were however, distinctly delayed.

The degranulation kinetics of CB-treated neutrophils stimulated with purified C5a and FMLP is shown in Figures 5 and 6. The release of all constituents was faster than that observed with either ZC or SaγG, and reached a maximum in 10 to 15 sec. The sequential pattern of LF, MPO, and β-glu seen with ZC and SaγG, almost disappeared. Yet, when the combined normalized data of two separate experiments was subjected to an analysis of variance (46), the release values for LF and MPO differed statistically at the earliest time points with a "p" value of <0.05. After 10 sec these values became indistinguishable. There was no such difference shown between the early release kinetics of β-glu and MPO.

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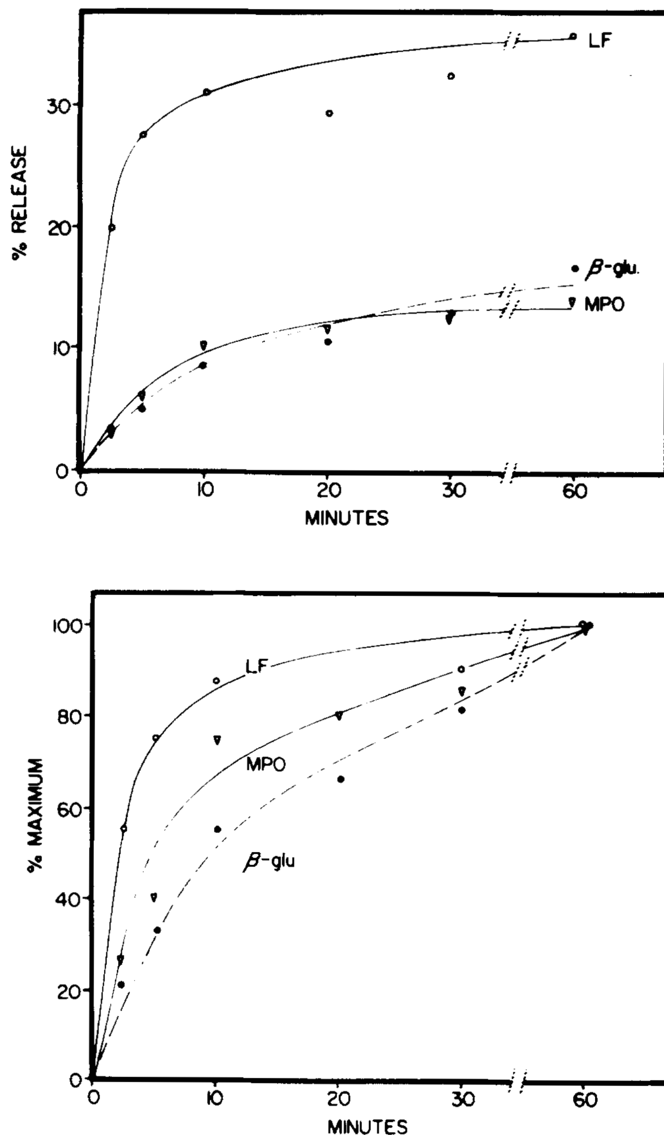

Figure 2. Kinetics of neutrophil granule release in response to ZC at 37°C. *A*, release as a percent of total; *B*, the percent maximal release.

By using a 10-fold higher dose of either purified C5a or FMLP, normal neutrophils (without CB) released up to 25% lys and 5% MPO within 2 min. Thus, although the extent of release was enhanced by CB pretreatment, neither the sequence nor the rate of granule release appeared altered.

Degranulation kinetics with PMA. The croton oil extract, PMA, has previously been described as a neutrophil stimulus that is selective in eliciting discharge of mainly the specific granules (28, 30, 36). However, as with other soluble stimuli, neutrophils pretreated with CB will respond to PMA with enhanced specific granule release *and* substantial azurophil granule release (47).

The response of CB-treated neutrophils to 10 ng of PMA is shown in Figure 7*a* and *b*. The kinetics were similar to those observed with C5a and FMLP except for the much longer reaction time required for response. Maximal LF release required 5 min with PMA, as opposed to 60 sec with C5a and FMLP. With PMA the sequential release pattern was again apparent, particularly with respect to β-glu release, which did not reach maximum until 15 min. MPO and β-glu showed different patterns of release, β-glu requiring a longer time course for maximal release.

DISCUSSION

Human neutrophils responded to soluble and particulate stimuli with a sequential release of first the specific granule constituents followed by those of the azurophil granules. The overall rate at which release ensued and the time required to achieve a maximal response varied depending upon the stimulus. Both the rate and extent of azurophil granule release exhibited marked differences in response to different types of stimuli. In all cases, azurophil granule constituents were released to the supernatant later or more slowly than those of the specific granules.

The rapid release of granule constituents from human neutrophils in response to soluble stimuli has made any previous demonstration of a sequential process difficult. Although lys served as an adequate specific granule marker when there was minimal azurophil granule exocytosis, as with PMA, Con A, or ionophore A23187, monitoring of lys release would not allow for a clear distinction between the release kinetics of the separate granule types because of its overlapping content in both. It was only by a comparison of the azurophil granule enzymes, MPO

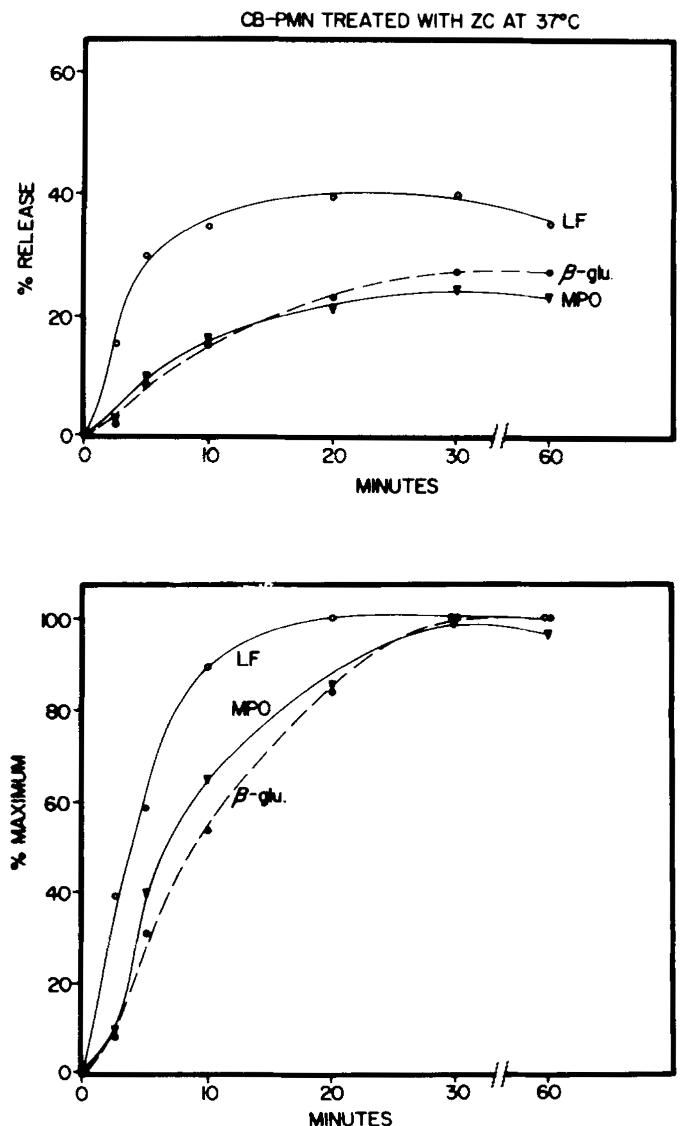

Figure 3. Granule release kinetics of CB-treated neutrophils (0.5 μg/ml) in response to ZC at 37°C. *A*, release as a percent of total; *B*, percent maximal release.

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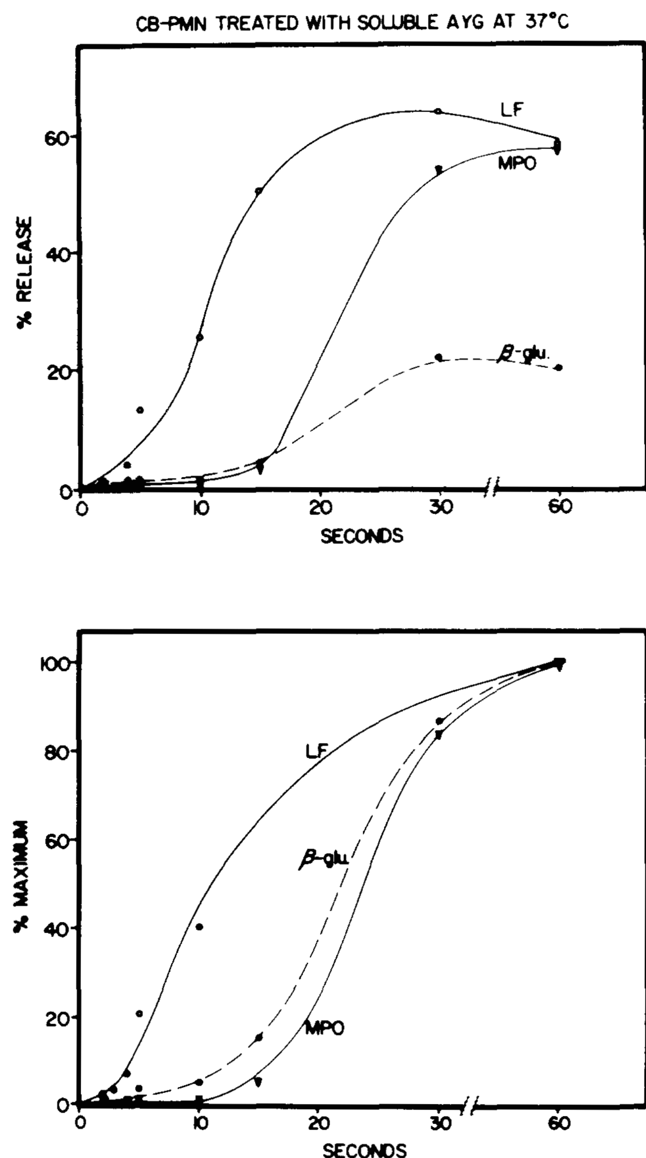

Figure 4. Granule release kinetics of CB-treated neutrophils (5 μg/ml) in response to SaγG at 37°C. *A*, release as a percent of total; *B*, percent maximal release.

and β-glu (19), with the exclusive specific granule protein, LF (20), that separate release kinetics could be clearly demonstrated.

To assay very early reaction time points, the Eppendorf centrifugation method presented proved to be the most efficient. This experimental procedure for incubation and pelleting of the cell suspensions required an elapsed time of less than 30 sec, from the start of the centrifugation after each incubation period until the supernatant was cooled to 4°C and removed. However, the cells were probably effectively separated from the supernatant much more rapidly than this. No release was detectable at the earliest time points and there was no leakage or breaking of the cells resulting in nonspecific enzyme release. We have reported release values at incubation times of 2 sec with C5a and FMLP. It should be noted that this time value represents the time from stimulus addition until centrifugation. However, because the release values of the unstimulated samples and the zero time samples were within 0.5%, any release during the processing time was insignificant.

The degranulation induced by the calcium ionophore, A23187, with 1.25 mM Ca^{++} required a 60-min incubation period to achieve maximal response and demonstrated a large differential between the time courses for lys and MPO release. In bypassing a stimulus-receptor interaction, as this ionophore is postulated to do, specific granules appear to be preferentially released over azurophil granules. We have also observed, as have Goldstein *et al.* (33), that increased external Ca^{++} concentrations, at this dose of ionophore, will markedly enhance the release of azurophil granule constituents but will only slightly increase the amount of lys released (unpublished observations). Cytoplasmic Ca^{++} mobilization appears to participate in the labilization of both granule types, but may be a critical component in triggering azurophil granule release.

CB-pretreated cells responded initially more slowly to ionophore, but the rate of release increased as the reaction proceeded, resulting in higher maximum values for both enzymes

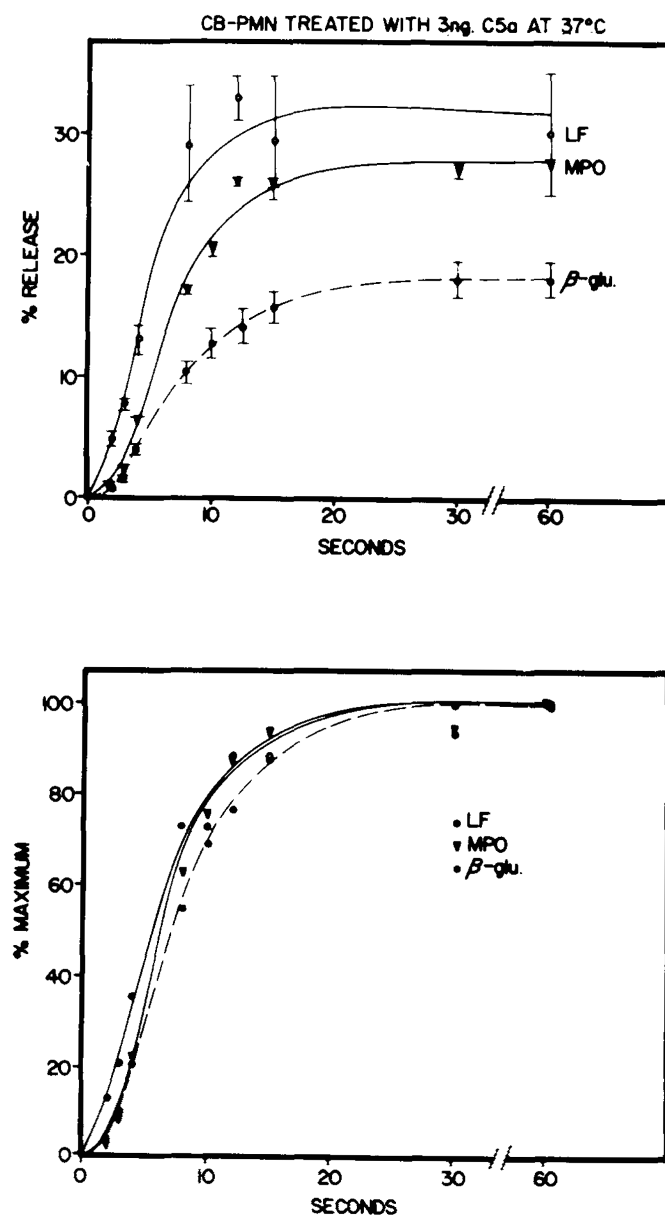

Figure 5. Degranulation kinetics of CB-treated neutrophils (5 μg/ml) stimulated with 3 ng/ml purified C5a at 37°C. Combined means of two separate experiments ± S.E.M. *A*, release as a percent of total; *B*, percent maximal release.

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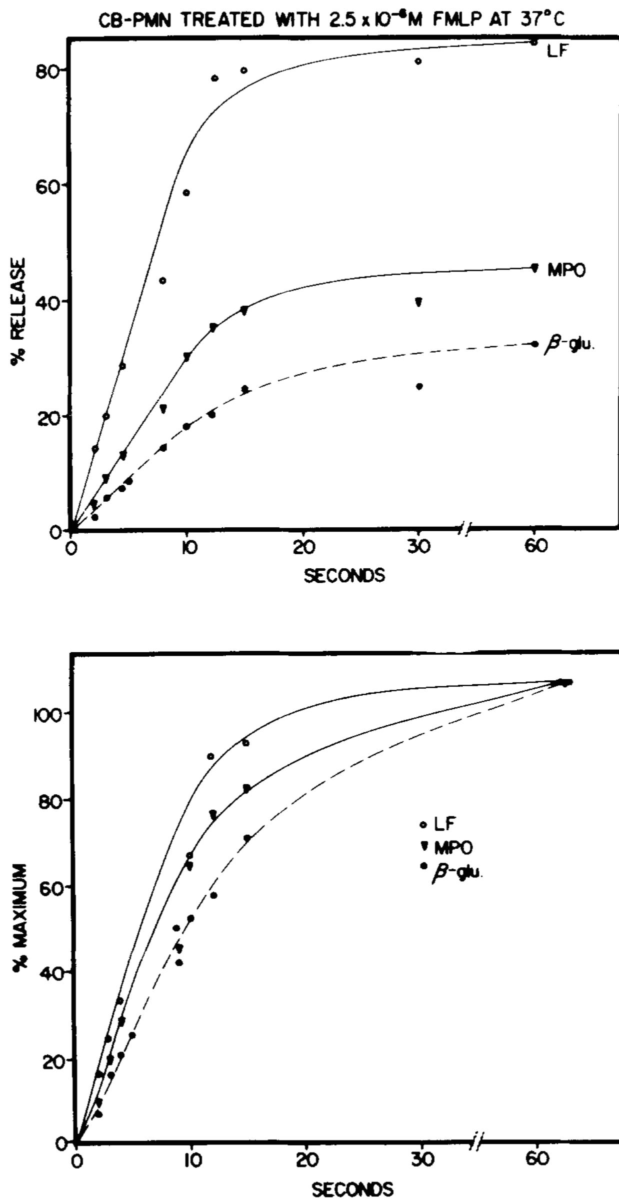

Figure 6. Kinetics of granule release from CB-treated neutrophils (5 μg/ml) stimulated with 2.5×10^{-6} M (FMLP) at 37°C. *A*, release as a percent of total; *B*, percent maximal release.

at 60 min. Maximal MPO release was augmented 7-fold after CB treatment, whereas lys release was enhanced only about one-third. CB pretreatment thus preferentially enhanced the amount of azurophil granule release with ionophore, as did increased external calcium concentrations, but the sequence of release was not affected.

ZC, a phagocytic stimulus, also required up to 1 hr to induce a maximal degranulation response from human neutrophils. A clear sequence pattern was observed with release of the specific granule contents, LF, followed by the azurophil granule constituents. Under these circumstances separate kinetic patterns could be distinguished for the release of MPO and β-glu, β-glu being released more slowly. Because the zymosan particles were being internalized, the observed sequence might have reflected differential solubilization or trapping of one or both of the azurophil granule components within the phagocytic vacuole, or binding of the enzymes to the zymosan. The longer time required for response may be due to the retarded pseudopod formation observed with this dose of CB (Henson and Henson, unpublished observations), which would limit the amount of cell surface area in contact with the stimulus. In the same experiment, neutrophils pretreated with CB were slower in responding to ZC, but demonstrated the same sequential release pattern as untreated cells, with a slight enhancement of specific granule release and a marked enhancement of azurophil granule release. It is unlikely, therefore, that the observed sequence is a reflection of preferential trapping of the auzrophil granule enzymes within vacuoles, but rather is evidence for a tertiary form of granule containing β-glu.

Degranulation from CB-treated neutrophils in response to soluble stimuli occurs directly into the supernatant in the absence of phagocytosis (11) and occurs within a time course of seconds rather than minutes as required for ionophore and ZC. In addition to a more rapid response, there was a greater percentage of azurophil granule release with the soluble stimuli. SaγG, acting through the surface Fc receptor, required a slightly longer incubation time to trigger a neutrophil response than did

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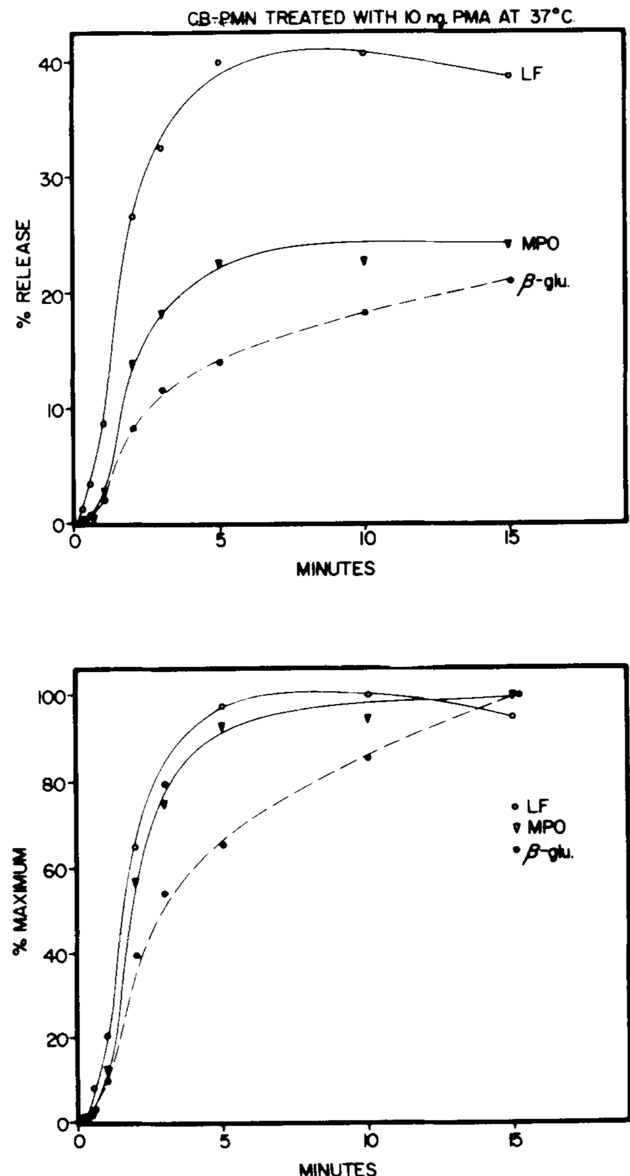

Figure 7. Kinetics of granule release from CB-treated neutrophils incubated with 10 ng PMA at 37°C. *A*, enzyme release as a percent of total; *B*, the percent maximal release.

C5a or FMLP. This may reflect the time requirement for a surface receptor modulation dependent upon the binding affinity and concentration of SaγG. Subsequent capping or cross-linking after binding may be required in order to trigger granule release. This stimulus also demonstrated a clear differential between the release of the specific and azurophil granule components. Again, specific granule release was rapid, reaching maximal values within 1 min, whereas the onset of azurophil granule release was delayed and took longer to reach a maximum.

C5a and FMLP, both potent stimuli for chemotaxis and degranulation, induced a rapid degranulation response from CB-treated neutrophils in these and previous studies (7, 42). With these stimuli, the speed of the initial response made a sequence difference for the release of the two granule types nearly indistinguishable. Only at the earliest time points could any statistically significant difference between the release rates be demonstrated. However, even in these cases, the sequence pattern was consistent, with the specific granule contents being released faster than those of the azurophil granules.

PMA as a soluble stimulus represents an example of how azurophil granule constituent release (the two state degranulation process) can be modulated. As previously demonstrated (47), PMA will induce a low but significant release of azurophil granule contents from CB-treated neutrophils. In the present study, we have demonstrated that this degranulation with PMA occurs with relative kinetics similar to those demonstrated by using C5a and FMLP.

Several mechanisms for the observed sequential release process can be postulated. First, Bainton and Farquhar (48) determined that human neutrophils contain a greater number of specific than azurophil granules. This quantitative difference might give the appearance of faster and greater specific granule release. Second, specific granules might directly trigger azurophil granule release either by providing internal membrane receptors for subsequent azurophil granule fusion or by the release of factors that stimulate azurophil granule mobilization. Third, azurophil granules may be less mobile within the cytoplasm, possibly due to their larger size or to more rigorous controlling factors, or the azurophil granule contents may solubilize more slowly into the external supernatant.

For any of these mechanisms, a consistent relationship between the individual granule-release kinetics, even with different stimuli, would be expected, based on inherent characteristics of each granule type or of the constituents therein. Yet, as discussed previously, there was not a direct correlation between the relative amounts or rates of granule constituent release from the two granule types. Azurophil granule constituents were released both slowly and rapidly depending upon the type of stimulus. Great differences were exhibited in the amount of release dependent upon pretreatment with CB. It is unlikely that the solubility of MPO into the supernatant would be a factor in this difference. Yet, with all the various stimuli tested, the sequential pattern was consistent.

It seems most reasonable to conclude that the mobilization and discharge of the specific and azurophil granules is triggered by separate intracellular steps or processes. This concept is supported by the observations of several investigators. Based on the selective release of specific granule associated enzymes by Con A (32), PMA (28, 30), and ionophore A23187 (36), and the leukocyte pyrogen (49), the exocytosis of specific granules has been correlated with a secretory role for neutrophils in the process of inflammation (49). Specific granule degranulation may then occur by a functionally independent process, not necessarily requiring phagocytosis and without the concomitant release of hydrolytic enzymes from the azurophil granules.

Alternatively, neutrophils may respond to various stimuli by a graded series of responses. These would include depolarization (50), an oxidative burst (34, 9), changes in intracellular calcium distribution (51, 52), cytoskeletal changes (11, 53, 54) and granule discharge. The degranulation sequence may then also be part of the graded series of responses. Either the release of specific granules alone, or specific granules plus varying amounts of azurophil granules may reflect different response levels to various stimulus parameters. Increased concentration for surface binding, particle size, or the degree of valency of soluble and surface-bound components might determine to what extent neutrophils will progress in a cascade of responses. Further characterization of such graded responses with human neutrophils remains to be elucidated.

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