Stimulation of Human Neutrophils by Soluble and Insoluble Immunoglobulin Aggregates

SECRETION OF GRANULE CONSTITUENTS AND INCREASED OXIDATION OF GLUCOSE

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ABSTRACT Reaction of human neutrophils with aggregated immunoglobulin on nonphagocytosable surfaces results in secretion of granule enzymes (exocytosis of granules) and stimulation of glucose oxidation by the hexose monophosphate pathway (HMP). The role of HMP stimulation in the enzyme secretion and some requirements for the two neutrophil activities have been examined. It was found that (*a*) HMP stimulation could be selectively inhibited under conditions where release of granule enzymes remained unchanged or was enhanced, for example, by reduced glucose concentration or by 2-deoxyglucose. (*b*) Removal of Ca^{++} and addition of agents which increased the intracellular levels of cyclic AMP (cAMP), however, prevented both activities, while colchicine had greater inhibitory activity on HMP stimulation than upon secretion. (*c*) Neutrophils incubated in suspension with particulate aggregated γ-globulin phagocytosed the particles and exhibited a stimulated HMP and released granule enzymes. In contrast, incubation in suspension with soluble aggregated γ-globulin resulted in the stimulated HMP only. Granule enzymes were not liberated. 300-fold less soluble aggregates bound to a surface, however, readily induced exocytosis of granules from adherent neutrophils. This demonstrates the importance of surface effects in the induction of secretion from neutrophils.

Aggregated immunoglobulin reacting with neutrophil Fc receptors thus induces both degranulation (exocytosis) and increased HMP activity. The pathways leading to these events are separable although apparently sharing some common steps, including the initiating events.

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INTRODUCTION

During the process of phagocytosis of immune complexes by neutrophils, fusion of cytoplasmic granule membranes with the resultant phagocytic vacuole allows liberation of granule constituents into the vacuole. This process has been referred to as degranulation. Recent evidence (1–4) has clearly shown that degranulation of human neutrophils results in a portion of the granule enzymes gaining access to the extracellular medium by processes which do not involve lysis of the cell. Various mechanisms whereby the vacuoles open to the outside have been demonstrated (1, 2, 5). A more direct way of examining degranulation, however, comes from studies with immunologic reactants bound to surfaces which cannot be phagocytosed (6, 7). The neutrophils adhere to the stimulus (e.g. antibody or complement) and have been shown to release granule constituents by direct exocytosis of the granules along that portion of the cell membrane which contacts the immune reactants (1, 8, 9). This process stimulates in vivo situations where neutrophils adhere to immune complexes on surfaces such as the glomerular basement membrane and release their granule enzymes, which then produce injury to the tissues (9). Attempts to determine the physiologic requirements for degranulation in phagocytosing neutrophils have been difficult since inhibitors of degranulation may also affect stages in the phagocytic process, e.g., the adherence or engulfment steps (10). Accordingly, the model employing exocytosis of granules along nonphagocytosable surfaces was adapted

to examine, in the absence of phagocytosis, the metabolic requirements and more specifically the role of the hexose monophosphate shunt in degranulation and release of potentially injurious constituents from neutrophils in inflammatory reactions. The experiments have also allowed a comparison to be made between the characteristics of two neutrophil activities, degranulation and stimulation of the hexose monophosphate shunt.

METHODS

Neutrophils. Venous blood was taken from normal human volunteers into 1/7 vol of acid citrate dextrose (11). Neutrophils were prepared by methods previously described (4, 6). The blood was centrifuged at 300 *g* for 20 min at room temperature. The platelet-rich plasma and buffy coat were removed and the remaining cells were resuspended in 2 vol of 2% gelatin in saline. The erythrocytes were sedimented at 1 *g* for 30 min at 37°C, the supernate removed, and the cells in it sedimented at 350 *g* for 10 min at room temperature. The remaining erythrocytes in this pellet were lysed by resuspending in 0.83% NH_4Cl pH 7.2 for 5 min at room temperature. The neutrophils were sedimented at 200 *g* for 10 min and washed once in the cold in Tyrode's solution buffered with 0.01 mol/liter Tris(Tris-hydroxymethyl amino methane) at pH 7.2 with 0.25% bovine serum albumin (BSA,[1] Pentex Biochemical, Kankakee, Ill.) and 5×10^{-3} mol/liter glucose. The cells were suspended at a concentration 2×10^7 neutrophils/ml in this solution and kept cold until used. Between 50 and 75% of the available neutrophils were recovered by the technique with a purity of from 96 to 98%.

Aggregated γ-globulin. Normal human γ-globulin (HGG, Pentex, Kankakee, Ill.) was aggregated with bis-diazotized benzidine (BDB), as described elsewhere (4), based upon the method of Ishizaka et al (12). In some experiments γ-globulin highly purified by *o*-(diethylaminoethyl)cellulose (DEAE-cellulose) column chromatography (4) was employed but the results obtained with either preparation were identical. Use of 25 μg BDB/mg HGG generally yielded macroscopic aggregates ("insoluble aggregates"). Lower concentrations of BDB were employed to yield clear solutions of aggregated γ-globulin ("soluble aggregates"). These remained soluble in all the buffers and with all the inhibitors used. For some experiments, γ-globulin was labeled with ^{125}I by the method of McConahey and Dixon (13).

Reaction with neutrophils. Two reaction conditions were employed. (*a*) Neutrophils incubated with immunoglobulin in suspension. Aggregated γ-globulin preparations were incubated with 4×10^6 neutrophils in 1.5 ml Tris-buffered Tyrode's solution containing 0.25% BSA for 30 min at 37°C with mild shaking. After centrifugation the sediment and supernates were assayed for β-glucuronidase and lactic dehydrogenase as described below. (*b*) Neutrophils incubated with immunoglobulin on the nonphagocytosable surfaces. Plastic petri dishes (60 × 15 mm, Falcon Plastics, Oxnard, Calif.) were incubated at room temperature for 60 min with 2 ml of 2.5 mg/ml aggregated γ-globulin (soluble aggregates) and then washed extensively with saline. Approximately 30–50 μg of aggregates remained firmly bound to the surface, and by using radiolabeled aggregates it was shown that more than 95% of these remained bound during the reaction with neutrophils. Control petri dishes were prepared with BSA. Neutrophils (4×10^6) were allowed to settle on the petri dishes in 1.5 ml medium and were incubated at 37°C for 30 min. At the end of the incubation period supernates were removed, centrifuged to sediment the few cells which do not adhere to the surface, and assayed for released enzymes. Total enzyme content was obtained by lysis of 4×10^6 cells or the cells remaining on the dish and in the pellet with 1 mg/ml Triton X-100. All reactions were performed in duplicate and the results expressed as a percentage of the total available enzyme. Alternatively, some experiments were performed with micropore filters (Millipore Corp., Bedford, Mass.) as the surface by techniques previously described (6).

For the adherence assays, neutrophils were labeled by incubation with 50 μCi ^{51}Cr (Na chromate) per 10^7 cells for 60 min at 37°C. They were washed and shown to behave normally in the functional assays. Incubation of the treated or control ^{51}Cr-labeled neutrophils on the petri dishes was followed by washing in warm buffer and finally elution of the bound cells with 1.0 M NaOH. Samples of the washes and eluates were counted.

Assays. The enzymes β-glucuronidase (E.C. 3.2.1.31), lysozyme (E.C.3.2.1.17), and lactic dehydrogenase (E.C. 1.1.1.27) were assayed as previously described (6). Lactate was measured according to the method described by Wieland (14) after extraction of the neutrophils with $HClO_4$. Glucose oxidation by the hexose monophosphate pathway (HMP) was assessed by the generation of $^{14}CO_2$ from [1-^{14}C]glucose (2, 15). Neutrophils were preincubated with [1-^{14}C]glucose for 1 h. For the experiments on Petri dishes 0.5 or 1.0 μCi labeled glucose was included with the neutrophils in Tyrode's solution containing only 1×10^{-3} mol/liter glucose (i.e., 1/5 of the usual concentration) to increase the specific activity.

Inhibitors. Dehydroepiandrosterone (Sigma Chemical Co., St. Louis, Mo.) was dissolved in methyl cellosolve and cytochalasin B (Imperial Chemical Industries, Alderley Park, Cheshire, U.K.) in dimethyl sulfoxide. Theophylline and colchicine (Sigma, St. Louis, Mo.), iodoacetate, azide, 2,4-dinitrophenol, antimycin A (Calbiochem, San Diego, Calif.), and 2-deoxyglucose were dissolved in saline. It was ascertained that the solvents themselves were without effect at the concentrations used. Inhibitors were preincubated with the neutrophils for 60 min at 37°C as well as being present during the reaction. Lactic dehydrogenase (LDH) release was always assayed to ensure that cell lysis was absent. Experiments with colchicine were carried out in Puck's saline G or in Tyrode's solution buffered with bicarbonate to avoid the inhibitory effect of Tris.

RESULTS

Release of granule enzymes; the effect of aggregate size. Human neutrophils were incubated in suspension with insoluble aggregates. The resultant release of enzymes is shown in Fig. 1. The granule enzymes β-glucuronidase and lysozyme were liberated from the cells reacting with insoluble aggregates. This release accompanied phagocytosis of the aggregates as observed by electron microscopy (4, 5). LDH was not released, indicating absence of cell lysis. Control prepa-

[1] *Abbreviations used in this paper:* BDB, bis-diazotized benzidine; BSA, bovine serum albumin; cAMP, cyclic AMP; HGG, human gamma globulin; HMP, hexose monophosphate pathway; LDH, lactic dehydrogenase.

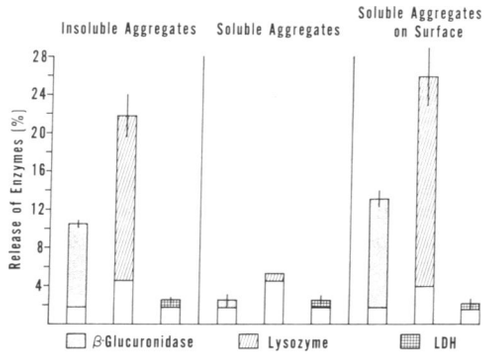

FIGURE 1 Release of enzymes from human neutrophils. (*a*) Washed neutrophils (4×10^6) incubated for 30 min at 37°C in suspension with 500 μg insoluble aggregates of human γ-globulin. The percent release±SEM is indicated. The nonshaded area of the columns represents the control release from neutrophils incubated alone. (*b*) Neutrophils incubated in suspension with 500 μg soluble aggregates. The control represents neutrophils incubated with non-aggregated γ-globulin. (*c*) Neutrophils (4×10^6) adherent to petri dishes, the surfaces of which have been coated with soluble aggregates. Controls (nonshaded areas) represent petri dishes with BSA on the surface.

rations of neutrophils incubated alone exhibited little release of constituents.

Neutrophils incubated in suspension with soluble aggregates on the other hand did not release granule enzymes. In contrast, the same soluble aggregates at-

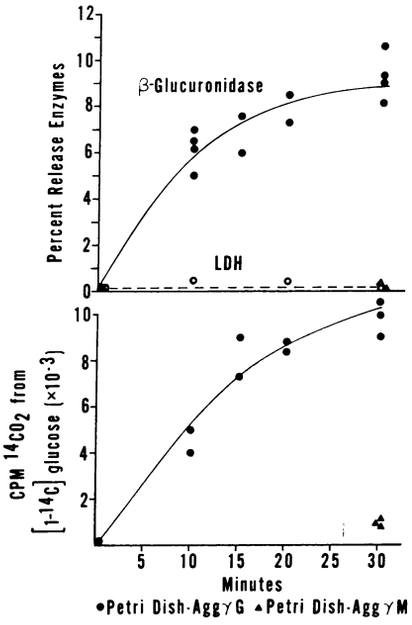

FIGURE 2 Stimulation of the HMP in neutrophils adherent to aggregated γ-globulin on surfaces. The percent release of β-glucuronidase is compared with the generation of $^{14}CO_2$ from [1-^{14}C]glucose (0.5 μCi per petri dish).

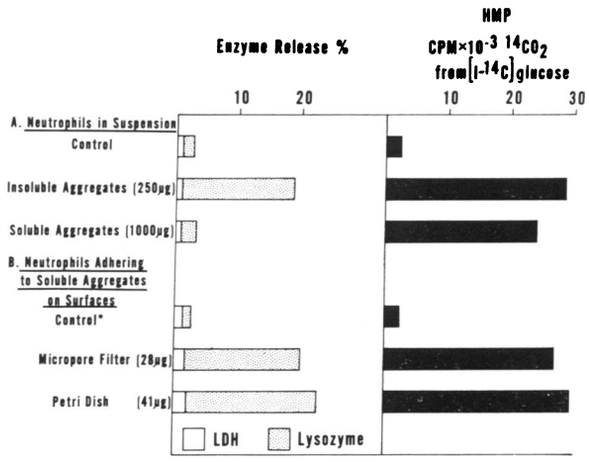

FIGURE 3 Release of granule enzymes and stimulation of the HMP by soluble and insoluble immunoglobulin aggregates. The amount of aggregate presented to the cells in suspension or upon the surfaces is depicted in parentheses. Control in A represents neutrophils incubated alone and in B, neutrophils adherent to BSA on the surface.

tached to a surface, even in very small amounts, initiated release of granule constituents from adherent neutrophils. This release, as indicated above, has been shown to result from extrusion of granules to the outside of the cell (1, 8). Control preparations of neutrophils on surfaces with BSA or aggregated IgM did not liberate the enzymes (Figs. 1, 2, and 3).

Stimulation of glucose oxidation through the HMP. Adherence of neutrophils to soluble aggregated γ-globulin bound to a surface stimulated the oxidation of glucose as determined by generation of $^{14}CO_2$ from [1-^{14}C]glucose (Fig. 2). The CO_2 generation was not prevented by KCN (1×10^{-3} mol/liter), indicating that the glucose was being oxidized by the HMP. A similar increase was seen during phagocytosis of insoluble aggregates (Fig. 3) and is a general accompaniment of phagocytosis.

Stimulation of the HMP without release of granule enzymes. Experiments were performed to determine whether the reaction of neutrophils in suspension with soluble aggregates (which did not result in release of enzymes) would nevertheless lead to stimulation of the HMP. Soluble aggregates (as little as 200 μg) were found to stimulate glucose C-1 oxidation in neutrophils in suspension. However, as shown in Fig. 3, 1,000 μg aggregates, which stimulated as much HMP activity as phagocytosis of insoluble particles, had no stimulatory action on secretion unless the aggregates were bound to a surface. Attempts were made to ascertain the amount of γ-globulin bound to the neutrophils in suspension using radiolabeled aggregates. Incubation of 1,000 μg soluble aggregates with 4×10^6 cells for

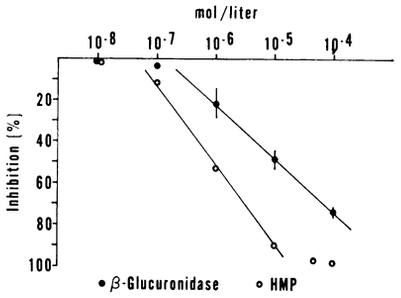

FIGURE 4 Inhibition by iodoacetate of release of β-glucuronidase and of stimulation of the HMP in neutrophils adherent to aggregated γ-globulin on petri dishes. The points represent percent inhibition of the effect in glucose-containing buffer±SEM.

30 min yielded 2 μg or less bound or taken up after the cells were washed.

The effect on enzyme release and HMP stimulation of inhibitors of cell energy metabolism. Experiments were performed to determine the involvement of different pathways of energy metabolism in the neutrophil activities. The release of β-glucuronidase from neutrophils on surfaces, as a marker for the granule enzymes, was markedly inhibited by iodoacetate (Fig. 4), 50% inhibition being achieved with 10^{-5} mol/liter, i.e., $pI_{50} = 5.0$. By contrast, KCN(1×10^{-3} mol/liter), antimycin A (10 μg/ml), and azide (1×10^{-3} mol/liter) did not inhibit this release.

The stimulation of glucose oxidation by the HMP was also inhibited by iodoacetate ($pI_{50} = 6.0$) at 1/10 the amount that was required for inhibition of enzyme secretion.

Inhibition of the HMP without prevention of enzyme release. A requirement for glycolysis. Stimulation of glucose oxidation by the HMP has in the past been linked to degranulation. Accordingly, experiments were performed to determine if release of β-glucuronidase from neutrophils on surfaces could be prevented by inhibition of either the HMP or glycolysis. Glycolytic activity was assessed by the production of lactate since generation of $^{14}CO_2$ from [6-^{14}C]glucose was found to yield only very low values and depends upon more than just the glycolytic pathway.

The effect of various inhibitors on release of β-glucuronidase, on glycolysis, and the HMP is shown in Fig. 5. All inhibitors were preincubated for 60 min with the neutrophils and included in the reaction. Iodoacetate, as described above, prevented release of the enzyme and inhibited both HMP stimulation and glycolysis. In contrast, 2-deoxyglucose, which is known to compete with glucose-6-phosphate for enzymes of both the glycolytic and HMP (16), in the presence of 10^{-3} mol/liter glucose did not inhibit, but rather enhanced the release of β-glucuronidase. It nevertheless produced a marked inhibition of the HMP, but only partially reduced the production of lactate. Dehydroepiandrosterone has been shown to inhibit glucose-6-phosphate dehydrogenase (17), the first enzyme of the HMP. As seen in Fig. 5, this steroid also inhibited the HMP without appreciable effect on release of enzyme or glycolysis. The steroid induced a dose dependent inhibition of the HMP, but even at 5×10^{-4} mol/liter (near the limits of solubility) had little effect on glycolysis. This may explain why enhancement of secretion was not seen with this agent.

The effect of 2-deoxyglucose in the absence of competing glucose is also shown. Marked inhibition of lactate production was now observed and concurrent with this, the release of β-glucuronidase was prevented. The control, incubation of neutrophils on surfaces with aggregated γ-globulin in the absence of glucose but with no inhibitor, was found to yield partial inhibition of glycolysis but a mild stimulation of enzyme release. This was similar to the stimulation seen when 2-deoxyglucose was used in the presence of competing glucose. Finally, removal of calcium with EGTA completely inhibited release of enzyme, HMP, and glycolysis. This effect required the preincubation for 60

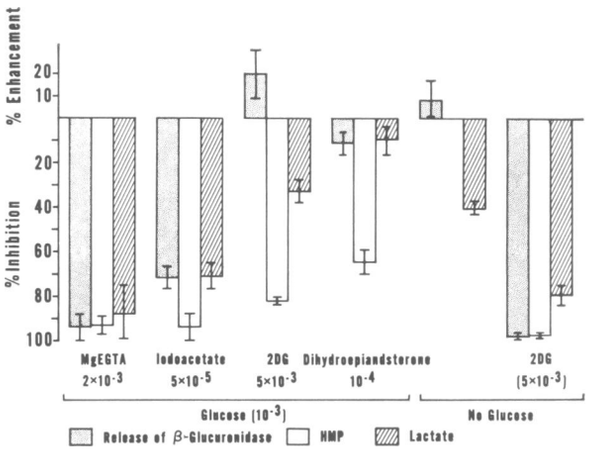

FIGURE 5 Effect of inhibitors on release of β-glucuronidase, production of lactate, and generation of $^{14}CO_2$ from [1-^{14}C]glucose (HMP) in neutrophils adherent to aggregated γ-globulin on petri dishes. Percent inhibition±SEM is shown below, and percent enhancement is shown above (the horizontal line). The concentration of the inhibitor used is shown in mol/liter. As depicted, 2-deoxyglucose was employed either with or without glucose in the medium. The control neutrophils incubated on aggregated γ-globulin without glucose has no HMP column shown because the greatly increased specific activity of the [1-^{14}C]glucose in this medium precluded comparison of the CO_2 generated with that in the other reactions. All inhibitors were preincubated with the neutrophils for 60 min at 37°C in addition to their inclusion during the reaction. Although not included in the figure, LDH release was at all times measured and was found to be negligible.

TABLE I

Binding of ^{51}Cr Neutrophils to Petri Dishes

		Number neutrophils bound $\times 10^{-5} \pm$SEM
Aggregated IgG-coated petri dish + 10^7 neutrophils		
No glucose	—	32.3±1.4
No glucose	$+5 \times 10^{-3}$ M 2-deoxyglucose	32.1±2.6
Glucose (10^{-3} M)	—	34.5±1.2
Glucose (10^{-3} M)	$+5 \times 10^{-3}$ M 2-deoxyglucose	34.1±1.8
Glucose (10^{-3} M)	$+10^{-4}$ M dehydroepiandrosterone	33.6±2.1
Glucose (10^{-3} M)	$+5 \times 10^{-5}$ M iodoacetate	34.6±1.4
Glucose (10^{-3} M)	$+2 \times 10^{-3}$ M EGTA	27.5±2.4
Glucose (5×10^{-3} M)		34.3±0.9
BSA-coated petri dish + 10^7 neutrophils		
No glucose		29.2±3.6
Glucose (5×10^{-3} M)		27.8±2.9

min of the human neutrophils with the chelating agent which was much less effective if merely included at the time of the reaction. The observation contrasts with that on rabbit neutrophils, release from which is more readily inhibited by EGTA (9).

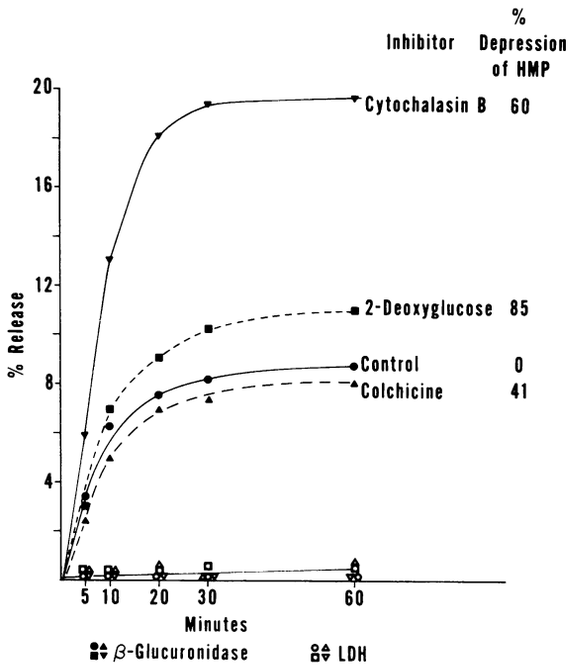

FIGURE 6 Time-course of release of β-glucuronidase and LDH from neutrophils reacting with aggregated γ-globulin on petri dishes in the presence or absence of enhancing or inhibiting agents. Colchicine and 2-deoxyglucose were preincubated with the neutrophils for 1 h before the reaction. The HMP activity was assayed after 60 min and is expressed as a percent inhibition of the control level. Each point represents the mean of duplicate determinations, and the experiment was repeated three times with similar results.

Table I depicts the effect of the inhibition or inhibitory conditions described in Fig. 5 on the adherence of neutrophils to surfaces with aggregated immunoglobulin. Only EGTA was found to have an effect, and that a mild one, on the adherence. This indicates that the inhibition or enhancement is not due to an effect on the numbers of neutrophils reacting with the stimulus.

Enhancement or inhibition with 2-deoxyglucose, cytochalasin B, or colchicine. Two deoxyglucose (1×10^{-3} mol/liter) in the presence of glucose (5×10^{-3} mol/liter) enhanced release of β-glucuronidase from neutrophils reacting with aggregated immunoglobulin on surfaces with the same time relationship as the control (Fig. 6). The 60-min incubation period allowed maximum release. Cytochalasin B, as previously described (18), demonstrated a much greater enhancement. Colchicine (1×10^{-4} mol/liter) was only marginally inhibitory. The colchicine induced 41% inhibition of HMP stimulation. HMP was also inhibited by the 2-deoxyglucose and by cytochalasin B (presumably because of inhibition of glucose uptake) (19). The above mentioned effects of 2-deoxyglucose and also of agents affecting microtubules and microfilaments were examined to determine if they represented a change in the time-course, as well as in the absolute amount of enzyme released.

The effect of glucose concentration on enhancement of enzyme release by cytochalasin B. If partial inhibition of glucose metabolism enhances release (see above), the enhancing effect of cytochalasin B may be related to its prevention of glucose uptake. As shown in Fig. 7, varying the glucose concentration over a

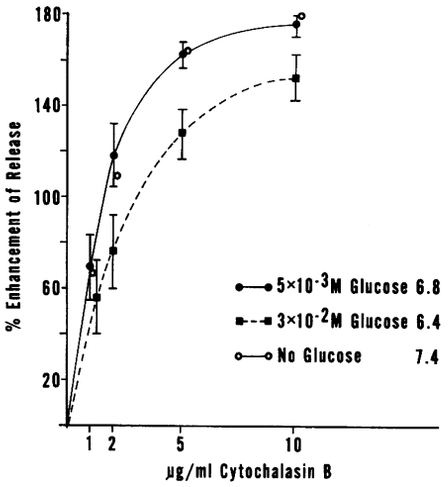

FIGURE 7 The effect of glucose concentration on the enhancement of release of β-glucuronidase by increasing concentrations of cytochalasin B. Neutrophils were preincubated with the glucose in Tyrode's solution for 1 h at 37°C and then allowed to adhere to aggregated γ-globulin on petri dishes in the presence of cytochalasin B. After each glucose concentration is depicted the percent release of enzyme measured in the absence of cytochalasin B. The agent did not induce release by itself and did not alter release of LDH which remained minimal.

large range only slightly affected the enhancement induced by cytochalasin. However, 3×10^{-2} mol/liter glucose did allow less enhancement by increasing concentration of cytochalasin B than 5×10^{-3} mol/liter. The figure also shows the dose range 0.5–5 μg/ml over which cytochalasin B is enhancing. Incubation of cytochalasin B alone with these neutrophils derived from human blood induced no release of enzymes in the absence of immunoglobulin stimulus. Although not shown, 2-deoxyglucose in the presence of glucose only slightly increased the cytochalasin effect.

The effect of theophylline on enzyme release and HMP stimulation. Agents which increase intracellular cyclic AMP (cAMP) in neutrophils (20–22) have been shown to inhibit release of granule enzymes from human and rabbit neutrophils phagocytosing zymosan particles or immune complexes (2, 3, 9, 21–25). The effect of one of these, the inhibitor of phosphodiesterase, theophylline, on release of enzymes and stimulation of HMP in human neutrophils adherent to aggregated immunoglobulin on surfaces was therefore investigated. Theophylline, in high concentrations, inhibited release of β-glucuronidase (Fig. 8). It also, with similar effectiveness ($pI_{50} = 3.3$) but different dose response, prevented stimulation of the HMP.

DISCUSSION

Release of granule enzymes from human neutrophils adherent to aggregated immunoglobulin (or C3) bound to nonphagocytosable surfaces has previously been demonstrated to result from exocytosis of granules along the stimulated membrane of the cell (1, 8, 9). This exocytosis of granules is similar to degranulation into a phagocytic vacuole, except that because of the size of the surface, the vacuole has not closed off. The system therefore serves as a model in which to examine the process of degranulation. We have now shown that glucose oxidation by the HMP was also stimulated by the reaction of neutrophils with aggregated immunoglobulins on surfaces. This stimulation does not, therefore, require the engulfment process since phagocytosis is prevented (6–8). Although both processes were initiated by the same stimulus (the γ-globulin) and were inhibited and controlled under similar conditions, they were nevertheless clearly dissociable by several criteria described below.

Dissociation of release of granule enzymes from stimulation of the HMP. Selective inhibition of the increased HMP activity was achieved with 2-deoxyglucose in the presence of competing glucose or with dehydroepiandrosterone, an inhibitor of glucose-6-phosphate dehydrogenase (17). Secretion of granule enzymes, however, was unaffected or enhanced. From the data presented, in the presence of glucose, 2-deoxyglucose apparently was a more effective inhibitor of glucose-6-phosphate dehydrogenase than of hexose phosphate isomerase (16), the first enzyme of the glycolytic pathway. This may reflect the much larger amount of glucose which is metabolized by glycolysis even during stimulation of the HMP (26). While the steroid may not only have been acting on glucose-6-phosphate dehydrogenase, its effect, nevertheless, was to prevent the HMP stimulation without appreciably inhibiting exocytosis of granules or glycolysis. In addition, stimulation of the HMP was inhibited by cytochalasin B, while granule exocytosis was enhanced by more than 200% (18, 23).

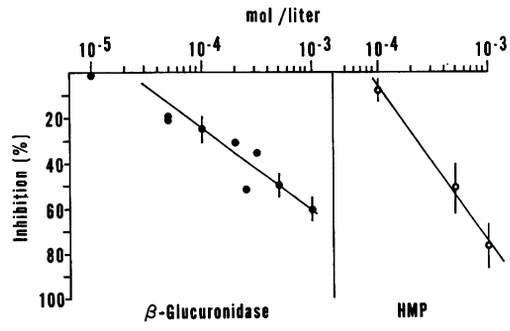

FIGURE 8 Inhibition by theophylline of release of β-glucuronidase and of stimulation of the HMP in neutrophils adherent to aggregated γ-globulin on petri dishes. The percent inhibition±SEM is given.

These observations, therefore, add direct evidence that the increase in glucose oxidation by the HMP is not an essential accompaniment of degranulation to that already suggested by studies of phagocytosis and bacterial killing (*27–29*).

In the reverse situation, HMP activity was stimulated by incubation of neutrophils with soluble aggregated γ-globulin without appreciable release of enzymes or phagocytosis. Similar stimulation of HMP with soluble immune complexes (30) or antineutrophil antibody (31) has been described. The observations suggest that in neutrophils oxidation of glucose by the HMP may be stimulated without overt degranulation. Thus, although HMP activity is induced by reaction of neutrophils with agents which also promote phagocytosis and degranulation, it is apparently not an essential participant in the processes of uptake and exocytosis. In like manner, uptake and exocytosis or degranulation are not necessary for stimulation of glucose oxidation. Nevertheless, stimulation of neutrophil membrane receptors by the Fc pieces of aggregated γ-globulin induces phagocytosis, exocytosis of granules (degranulation), and stimulation of the HMP, perhaps involving in part a common pathway which is blocked by removal of calcium or addition of theophylline or PGE_1.

Requirement for glycolysis, for granule secretion, and HMP stimulation. Iodoacetate, or 2-deoxyglucose in the absence of competing glucose, prevented the release of β-glucuronidase and also markedly inhibited glycolysis, as assessed by lactate production. These observations, together with the lack of effect of inhibitors of oxidative phosphorylation, strongly suggest that the glycolytic pathway supplies the energy metabolism requirements for the exocytosis. It is of interest that inhibition of glycolysis to only a slight degree was accompanied by marked inhibition of the HMP stimulation. However, a causative effect here, e.g. a reduced supply of oxidized NADP to "drive" the HMP (*26, 32*), seems unlikely since most of the inhibitory agents used have a direct effect on the HMP.

Iodoacetate was effective in preventing granule exocytosis on surfaces at concentrations well below those required to prevent uptake of bacteria (33) or latex particles (34). However, Bodel and Malawista (33) demonstrated that although uptake was not prevented, degranulation was reduced in the presence of this agent. They postulated the action of preformed metabolites (ATP) in uptake. These observations taken together suggest perhaps a greater requirement for ATP in the degranulation phenomenon than for phagocytosis.

Enhancement and inhibition of exocytosis and HMP stimulation. Enhancement of enzyme release was observed under conditions in which glycolysis was partially (but not totally) inhibited, while HMP activity was completely prevented. The reason for this effect is as yet unknown. We have previously described (18), as have others (*23, 35*), that cytochalasin B markedly enhances the release (exocytosis). This agent has also been reported to inhibit glucose uptake (19) and presumably because of this, reduces HMP stimulation (18, 36). The possibility that its enhancing effect was related to interference with glucose metabolism was not borne out in the experiments described herein. The corollary, however, that partial inhibition of glycolysis partially interferes with microfilament activity (thus mimicking the effect of cytochalasin B) may well be the case.

As with many cell activities (10) removal of calcium completely prevented the release of granule enzymes and the HMP stimulation. To effect complete inhibition of release from the human neutrophils studied here, preincubation with EGTA was required. This contrasts with observations on rabbit neutrophils (9) where addition of EGTA at the beginning of the reaction was sufficient. Prolonged incubation with EGTA presumably depletes intracellular Ca^{++} which for the human cell provide more of the Ca^{++} required for the secretory process. Microtubule function has been reported to be required for degranulation and for extracellular release of enzymes (*23, 37, 36*). In our hands, however, colchicine was only slightly inhibitory even though the same material, in concurrent experiments, completely prevented secretion from platelets and in the neutrophils did inhibit HMP stimulation, as previously described by others for HMP stimulation during phagocytosis (36). This discrepancy is at present unexplained but a similarly poor inhibitory effect was observed by Hawkins (38). It may relate to differences between neutrophils in suspension and neutrophils spread out on flat surfaces (See also reference 39). In this latter circumstance, reduced requirement for spatial organization within the cytoplasm may allow more ready movement of those granules which will be discharged, to the cell membrane, even in the absence of microtubules.

The modulating effects of cyclic nucleotides on cell functions, including those of neutrophils, are now well demonstrated (*25*). Theophylline was here shown to inhibit both secretion and HMP stimulation, but interestingly, with different dose response characteristics. In other experiments not reported, PGE_1 and dibutyryl cAMP were also shown to inhibit both responses. The data again suggest that different pathways are stimulated within the neutrophil but that each is nevertheless under cyclic nucleotide control.

Stimulation of neutrophils by aggregated γ-globulin. The experiments reported here demonstrate that human neutrophils were stimulated by immunoglobulin to pha-

gocytose particulate aggregates and release granule enzymes to the external medium. Soluble aggregated γ-globulin, on the other hand, stimulated increased glucose oxidation (HMP), but did not induce release of enzymes and only minor uptake of aggregates occurred. The same soluble aggregates, in much lower quantity, (less than 1/300) when bound to a surface actively induced both exocytosis of granules and HMP-stimulations. This figure is calculated from the data in Fig. 3. 28 μg soluble aggregates were bound to the filter. However, the neutrophils could only have been in contact with at the maximum 2.8 μg of bound immunoglobulin since the cells occupy only the center portion of the filter and penetrate less than one-fourth of the way into the filter, while the immunoglobulin is bound throughout. The observations suggest that particulate material is needed for active phagocytosis and degranulation (see also reference 26). They also suggest either that some configurational change in the cell membrane induced by a surface or particle is needed for uptake and degranulation as well as the reaction of γ-globulin with the cell membrane receptors, or that a high binding affinity is required, which in this case is supplied by the cooperative binding effects of many immunoglobulin molecules on the particle or surface.

Stimulation of two separable activities in neutrophils by one stimulus is not unique to immunoglobulin. We have recently shown that chemotactic factors induce degranulation (exocytosis) (40), and they also stimulate the HMP (40, 41) as well as promote chemotaxis. Recent evidence (Henson, unpublished work) also suggests that these factors are capable of stimulating phagocytosis (engulfment) if presented to the neutrophil on a suitable particle. It is therefore suggested that stimulation of neutrophil receptors may initiate one or more of a number of pathways in the cell which have common and unique steps and which result in different cell activities.

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