NEUTROPHILIC LEUKOCYTES IN IMMUNOLOGIC REACTIONS: EVIDENCE FOR THE SELECTIVE RELEASE OF LYSORSAMAL CONSTITUENTS¹

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The release of lysosomal bioactive factors is essential to the role of the neutrophilic leukocyte as a mediator of tissue injury in immunologic inflammatory lesions. The lysosomal release process was studied by exposing rabbit neutrophils to phagocytosable immune complexes, non-phagocytosable immune complexes and anti-neutrophil antibody and measuring the kinetics of release of a lysosomal marker enzyme (β-glucuronidase). All three types of immunologic stimuli produced a rapid, selective release of the lysosomal marker. This release preceded that of a cytoplasmic marker enzyme (lactic dehydrogenase) and was not associated with a general increase in cell membrane permeability as judged by trypan blue dye exclusion. In contrast when neutrophils were exposed to a cytotoxic chemical, N-ethylmaleimide, the opposite pattern was observed. Lactic dehydrogenase was rapidly released while release of β-glucuronidase was greatly delayed. These studies indicate that there is a non-cytotoxic, non-cytolytic mechanism for the immunologically induced release of neutrophil lysosomal bioactive factors. This process may be similar to the non-cytolytic release mechanisms described for other cells such as platelets and mast cells.

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Neutrophilic leukocytes (neutrophils) have been shown to play a major role in two apparently different types of process: host protection against microorganisms, and the mediation of host tissue injury in certain inflammatory lesions. The latter are particularly well exemplified by the group of experimental immunologic disorders that includes the Arthus phenomenon, serum sickness arteritis and acute nephrotoxic nephritis (1). In both the protective and destructive processes, the release of lysosomal constituents ("degranulation") appears to be essential. Inactivation of pathogenic organisms is effected by the release of antibacterial factors into phagolysosomes (2, 3) while the function of the neutrophil as a mediator of tissue injury in immunologic inflammatory conditions depends on the extracellular release of lysosomal constituents that are capable of increasing vascular permeability (4, 5), disrupting vascular structures (6), promoting coagulation (7) and perhaps triggering kinin formation (8).

At least two distinct mechanisms have been described whereby an immunologic stimulus may induce the release of neutrophil bioactive lysosomal factors: 1) the phagocytosis of immune complexes (5, 9) and 2) surface contact with non-phagocytosable immune complexes (10). Both mechanisms have been delineated in vitro but they do have their in vivo counterparts (1). It has been suggested that this release of neutrophil lysosomal factors in immunologic reactions is a relatively non-specific, non-selective process that reflects a general increase in cell membrane permeability (10), if not simply a regressive or degenerative cellular phenomenon (5).

The present experiments were designed to examine the selectivity and specificity of neutrophil lysosomal enzyme release in response to immunologic reactions in vitro. These studies showed that neutrophil lysosomal enzymes are selectively released extracellularly before the occurrence of a general increase in cell membrane

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permeability in all three immunologic reactions tested, namely, phagocytosis of immune complexes, contact with non-phagocytosable immune complexes and interaction with anti-neutrophil antibody.

MATERIALS AND METHODS

Neutrophilic leukocytes. Peripheral blood neutrophils were obtained from New Zealand rabbits as described previously (9, 10) by a modification of the method of Chodirker et al. (11). Red blood cells were removed by hypotonic lysis, and other white blood cells were largely eliminated by differential centrifugation. The final preparation contained more than 85% neutrophils. Preparations containing more than 5% non-viable cells, as judged by trypan blue exclusion, were discarded. In any one experiment, cells from a single rabbit only were employed.

Antisera. Antibody to horse spleen ferritin was produced as described previously (10). The immune serum yielded 20 mg antibody protein/ml by the quantitative precipitin test. The antibody was employed as the IgG fraction obtained by precipitation with ammonium sulfate and diethylaminoethyl cellulose chromatography. This fraction yielded a single precipitin line when made to react against sheep anti-rabbit normal serum in immunodiffusion and immunoelectrophoresis. Rabbit anti-bovine serum albumin (BSA) was produced and quantitated in a similar manner and contained 20 mg antibody protein/ml. Anti-rabbit neutrophil antisera was produced in guinea pigs by repeated subcutaneous injections of purified rabbit peripheral blood neutrophils in incomplete Freund's adjuvant. Anti-neutrophil antisera were titered by incubating the antisera at various dilutions with 10 × 10⁶ rabbit neutrophils in the presence of 10% fresh guinea pig serum in a reaction volume of 2.5 ml. Cells were incubated at 37°C for 30 min with this mixture and cytotoxicity evaluated by trypan blue dye exclusion. Unless otherwise indicated, antisera were used at a dilution which yielded 50% cytotoxicity at 30 min. The guinea pig anti-neutrophil antisera were employed in an unabsorbed form. They did not contain antibodies to rabbit serum proteins when tested by immunodiffusion and immunoelectrophoresis.

Collagen polymer membranes. Biosynthetic collagen polymer membranes were prepared as described previously (10). Basically, 0.5 ml of cadmium-free horse spleen ferritin (Pentex Biochemicals, Kankakee, Ill.), 30 mg/ml in 0.1 M acetic acid, was mixed thoroughly with 3.0 ml of acid-soluble calf skin collagen (Calbiochem, Los Angeles, Calif.), plated out in 55-mm diameter polystyrene dishes and allowed to dry slowly at room temperature. The dried membranes were strengthened by heating at 110°C for 12 hr.

Neutrophil response to immune reactants. In all experiments, except those employing anti-neutrophil antisera, cell concentrations were adjusted to 50 × 10⁶ neutrophils per tube or chamber in reaction volumes of 2.5 ml. Interaction with phagocytosable immune complexes in precipitate form was performed as described previously (9). Cells at 0°C were brought into a smooth suspension in Puck's saline G (Grand Island Biological Company, Grand Island, N. Y.) containing the immune precipitates. Ten per cent autologous heparinized plasma was added to the incubation mixture. The amount of antibody employed in complex form was 5000 μg antibody protein per 50 × 10⁶ neutrophils. Tubes containing the cell reaction mixture were covered and incubated for varying times at 37°C with constant agitation. At the end of the incubation period, tubes and their contents were rapidly cooled to 0°C, centrifuged at 325 × G for 10 min and the supernatant medium was separated from the cell pellet. Neutrophil interaction with non-phagocytosable immune complexes was carried out as described previously (10). Each collagen-ferritin biosynthetic membrane was preincubated with rabbit anti-ferritin antibody containing 10 mg antibody protein at 37°C for 45 min. The excess fluid was discarded and 50 × 10⁶ neutrophils added in 2.5 ml of Puck's saline G. The cells were permitted to interact with the antibody-coated membrane at 37°C for varying periods of time. At the end of the incubation period, the ambient medium from each chamber was decanted, cooled to 0°C and centrifuged at 325 × G. Neutrophil interaction with anti-neutrophil antibody was accomplished essentially as described under cytotoxicity evaluation. 1.5 × 10⁶ neutrophils were incubated for 3 min at 37°C and then anti-neutrophil antibody plus fresh guinea pig serum in a final concentration of 10% was added. The cell reaction mixture was incubated for varying times at 37°C without agitation. At the end of the incubation period, the mixture was centrifuged at 325 × G after rapid cooling to 0°C and the supernatant saved.
Figure 1. Release of neutrophil enzymes in response to phagocytosable immune complexes. Mean values and range of four experiments are shown. Beta glucuronidase is expressed as μM phenolphthalein generated in this and succeeding figures and tables.

Neutrophil-N-ethylmaleimide reaction. Neutrophils were exposed to 5 mM N-ethylmaleimide for varying times at 37°C in the presence of 10% autologous plasma. Neutrophil concentrations were adjusted to $1.5 \times 10^6$ cells per tube in a volume of 2.5 ml. Cell preparations were handled in a manner identical to that used in neutrophil anti-neutrophil antibody experiments.

Enzyme studies. The supernatant fluids obtained from each of the neutrophil-immune react- ant and neutrophil-N-ethylmaleimide mixtures were assayed for β glucuronidase by the method of Fishman et al. (12) and lactic dehydrogenase by the method of Bergmeyer (13). Beta-glu- curonidase and lactic dehydrogenase (LDH) were employed as lysosomal and cytoplasmic markers respectively. In certain experiments, freshly pre- pared neutrophilic leukocytes were separated into granule and cytoplasmic fractions as de- scribed previously (9) and assayed for total β glucuronidase and lactic dehydrogenase content.

Morphologic studies. At the end of the incubation period, each cell preparation was examined by phase microscopy. Cell viability was evaluated by trypan blue dye exclusion.

RESULTS

Neutrophil response to phagocytosable immune complexes. Exposure of neutrophils to phago- cytosable antigen-antibody complexes in pre-
Neutrophil response to non-phagocytosable immune complexes. Neutrophils responded to non-phagocytosable immune complexes on a biopolymer membrane with a virtually totally selective release of β glucuronidase. The results are seen in Figure 3. At 60 min the release of the lysosomal marker was comparable to that seen with phagocytosis of immune complexes, however, the release proceeded at a somewhat slower rate. As has been noted previously (10) minimal release of either enzyme occurred when neutrophils were exposed to the biopolymer membrane in the absence of specific antibody. These control values have been subtracted from the data in Figure 3.

Elimination of 10% autologous plasma from the incubation mixture did not change the release response significantly. The values in the plasma-free system are shown as open symbols in Figure 3. Application of greater quantities of antibody or more cells to the membrane did not increase the extent of release beyond that noted in Figure 3. The adherence of reactive neutrophils to the antibody-coated membrane precluded estimation of trypan blue uptake and, therefore, cytotoxicity could not be evaluated by this means.

Neutrophil response to anti-neutrophil antibody.

Initially neutrophils were reacted with heterologous anti-neutrophil antiserum. Ab = guinea pig antirabbit neutrophil antibody; GPS = fresh guinea pig serum; ΔGPS = guinea pig serum heated at 56°C for 30 min, BGL = β glucuronidase; LDH = lactic dehydrogenase.

Initially neutrophils were reacted with heterologous anti-neutrophil antiserum in the presence of complement in order to observe the effects of a cytolytic agent on the cell. It was anticipated that the antibody and complement would induce a rapid loss of the cytoplasmic marker, LDH, with a much more delayed release of lysosomal contents. While a cytotoxic effect was eventually observed, an unexpectedly rapid and substantial release of the lysosomal marker β glucuronidase was noted which preceded the release of LDH and the increased permeability of the cell to trypan blue. The results are shown in Figures 4 and 5. Elimination of the antibody or use of heat-inactivated guinea pig serum abolished both the early lysosomal release phase and the later “cytotoxic” effect. Heating of the antibody at 56°C for 30 min had no observable effect in this system. The early release of β glucuronidase occurred completely independently of LDH as seen in Figure 5. Within 3 min of exposure to antibody at a 1:80 dilution, marked β glucuronidase release occurred which approached 100% of that seen at 60 min. In contrast, maximum cytoplasmic marker release was seen only after 30 to 60 min (Fig. 4). Furthermore, at the time of near maximal β glucuronidase release, i.e., 3 to 5 min, 94% of the cells remained trypan blue impermeable whereas at 60 min 44% were permeable to the dye. The only morphologic change noted
at the time of the selective $\beta$ glucuronidase release was some swelling of the neutrophils.

When sufficiently high concentrations of antineutrophil antibody were employed, e.g., a 1:10 dilution, a rapid “cytotoxic” effect was observed in 5 min with simultaneous release of near maximal quantities of $\beta$ glucuronidase and LDH. The results are shown in Table I. At antibody dilutions up to 1:20, early release of large quantities of both the cytoplasmic and lysosomal markers was noted, however, at the higher dilutions rapid release of cytoplasmic marker enzyme was not observed despite virtually maximal early release of the lysosomal marker enzyme.

**Neutrophil response to N-ethylmaleimide.** The unexpected response of neutrophils to “cytotoxic” antibody, i.e., the selective early release of the lysosomal marker, led to a search for an agent that would produce the opposite effect, namely, early release of the cytoplasmic marker with delayed release of lysosomal enzyme. The search was undertaken primarily to demonstrate that independent selective release of LDH was possible and that the selective lysosomal release that had been demonstrated with the immune reactants was not simply a universal neutrophil response to any stimulus or toxic agent. The compound N-ethylmaleimide was shown to produce such an effect as seen in Figure 6. It was observed that maximal LDH release occurred well before any significant $\beta$ glucuronidase release and that the former correlated well with the uptake of trypan blue by the neutrophils. At 20 min 67% of the cells were permeable to the dye. N-ethylmaleimide did not interfere with either of the enzyme assays and had no effect on $\beta$ glucuronidase activity even after 90 min exposure. It did reduce LDH activity after 30 min exposure as noted in Figure 6, but it did not prevent observation of the early selective release of this enzyme.

**Neutrophil enzyme content.** Neutrophils were disrupted ultrasonically in 0.34 M sucrose at 0°C and the fractions treated with Triton-X 100 (9). Quadruplicate experiments gave mean values of 31 $\mu$M phenolphthalein/10$^7$ cells/hr for $\beta$ glucuronidase and 525 units/10$^7$ cells/hr for lactic dehydrogenase.

## DISCUSSION

The present experiments demonstrate that rabbit neutrophilic leukocytes will respond to a number of immunologic reactants in vitro with the selective release of lysosomal constituents. Evidence for the release of lysosomal enzymes was obtained by measuring a lysosomal marker, the enzyme $\beta$ glucuronidase, while general increase in cell permeability or “cytotoxic” effect was evaluated by assaying for the release of the cytoplasmic marker enzyme, LDH. In addition, in certain experiments cell membrane permeability or “cytotoxicity” was evaluated by measuring the uptake of the dye trypan blue into the cells.
The most convincing evidence for selective release of lysosomal constituents was obtained when the neutrophils were exposed to non-phagocytosable immune complexes in the form of an antibody-coated biopolymer membrane. In this situation, there was virtually exclusive release of β-glucuronidase with negligible release of the cytoplasmic marker even after 60 min incubation. The nature of the system made trypan blue uptake difficult to assess. The minimal release of LDH would seem to preclude a cytotoxic or cytolytic effect.

Release of the lysosomal marker was not diminished by elimination of fresh plasma from this system. Preliminary evidence suggests that plasma is necessary, however, if the antibody-globulin is employed in the form of (Fab')2 fragments (Hawkins, unpublished observations). Essentially identical results have been obtained by Henson who employed a non-phagocytic system where the antigen was mechanically entrapped in a Millipore filter prior to the application of antibody and cells (14). His system differed from the one used here in that marked internally convex deformation of the cell membrane occurred in a manner very similar to that seen in phagocytosis of immune complexes except that a true phagocytic vacuole was not formed. The importance of this mechanical event in release process is not clear at present. The spreading of neutrophils on a planar surface as seen with the antibody-coated polymer membrane (10) may represent an identical process, i.e., "frustrated phagocytosis." In this case the spherical object to be taken up has reached infinite size.

In a second system examined, the neutrophils were exposed to phagocytosable immune complexes in the form of antigen-antibody precipitates prepared at equivalence. In this system, rather marked LDH release did occur. The release, however, was gradual over the 60 min of the incubation period while release of β-glucuronidase was more rapid achieving a maximum shortly after 15 min of incubation. The release of LDH in this system was not associated with uptake of the dye trypan blue by a high percentage of cells for reasons that are presently unknown. Henson had also shown selective release of β-glucuronidase to the exclusion of LDH in immune phagocytic system when using zymosan particles coated with complement and latex particles coated with IgG (14).

In contrast to the minimal effect of plasma removal on the non-phagocytic system elimination of plasma from neutrophils exposed to phagocytosable immune complexes resulted in a marked decrease in the amount of specific release of both β-glucuronidase and LDH. As pointed out earlier, however, control values for cells incubated in a plasma-free system were considerably higher than plasma containing controls and, hence, a much larger background was subtracted.

It is customary to express release of cell constituents as a per cent of the total cellular content. This method was not employed in the present paper for the following reasons. In the first two systems examined, the cellular response to phagocytosable and non-phagocytosable immune complexes, release of β-glucuronidase seldom exceeded 7 μM/10^7 cells/hr. Varying the immune reactant-cell ratio did not increase this release. Furthermore the release appeared to reach a maximum and level off within 60 min or sooner. This suggests that the β-glucuronidase capable of being released in these two systems is probably not much greater than 7 μM/10^7 cells/hr even though disruption of the cells by physical-chemical means can liberate up to 30 μM/10^7 cells/hr of β-glucuronidase activity. Similarly, LDH values seldom exceeded 140 units/10^7 cells/hr even though up to 500 units/10^7 cells/hr LDH activity could be liberated by sonification and Triton X-100 treatment of the cells. Anti-neutrophil antibody and N-ethylmaleimide on the other hand released 100% of the "available" LDH but only 50% of the "available" β-glucuronidase. Because of the discrepancy in the extent of release when employing physiologic methods, i.e., immune reactants, vs physical-chemical disruption, it was felt that expression of the data as a per cent of total cell content would be somewhat misleading. The reason for the discrepancy is not clear at the present time. It may simply be that the physical disruption of the cells is more thorough or perhaps a certain percentage of the enzymes, especially the lysosomal ones, are incapable of being released by physiologic means because of binding (14) or insolubility.

Since neither the phagocytosable nor non-phagocytosable system seemed to operate through an immediate cytotoxic effect, an attempt was made to develop a cytotoxic system for comparative purposes. Neutrophils were incubated with guinea pig anti-neutrophil antiserum in the presence of 10% fresh guinea pig serum. Surprisingly,
TABLE I
The response of neutrophils to anti-neutrophil antiserum: the effect of antibody concentration on the release of intracellular enzymes

<table>
<thead>
<tr>
<th>Antibody Concentrationa</th>
<th>Enzyme Released</th>
<th>Lactic dehydrogenasec</th>
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<tr>
<td></td>
<td>β Glucuronidaseb</td>
<td>5 min 60 min</td>
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<tr>
<td></td>
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<td>5 min 60 min</td>
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<td>10</td>
<td>21.7</td>
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<tr>
<td>20</td>
<td>20.5</td>
<td>21.1</td>
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<tr>
<td>40</td>
<td>20.2</td>
<td>20.4</td>
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<tr>
<td>80</td>
<td>19.4</td>
<td>19.4</td>
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a Reciprocal of dilution.
b Expressed as μM phenolphthalein/10^7 cells/hr.
c Expressed as units/10^7 cells/hr.

The response of the cell was a rapid, striking release of the lysosomal marker with a much more delayed release of the cytoplasmic marker. Furthermore, the near maximal release of the lysosomal marker achieved with a few minutes of exposure to the antibody and complement source was not associated with uptake of trypan blue by the cell. This suggests that the lysosomal release preceded any immediate cytotoxic effect from the antibody and complement. Only after 45 to 60 min incubation did release of LDH approach maximal levels. At this time, trypan blue uptake by the cells also became evident. Both the cytotoxic effect and the early specific lysosomal release effect were dependent on the antibody and a fresh complement source. The use of normal guinea pig serum in the absence of antibody gave a minimal response while employment of the antibody with heated guinea pig serum also gave little or no response. As seen in Table I, the rapid cytotoxic effect was dependent on antibody concentration. It could be achieved with high antibody concentrations but tended to disappear at lower ones while the rapid release of β glucuronidase appeared independent of antibody concentration over a wide range of antibody employed. It would appear that two different release mechanisms may be operative when neutrophils are exposed to heterologous anti-neutrophil antibody in the presence of a complement source.

Since these three rather distinct immunologic reaction systems all produced an early selective release of β glucuronidase with a delayed or diminished release of LDH, it was possible that this represented an almost universal response of the neutrophil to any kind of stimulus and that it was not specific for immunologic reactions. An effort was therefore made to produce a pure cytotoxic effect by chemical means. Previous studies by Woodin and Wieneke (15) suggested that the compound N-ethylmaleimide retarded the extrusion of granule protein from neutrophils which were exposed to the bacterial product, leukocidin. Exposure of neutrophils to 5 mM N-ethylmaleimide produced a striking cytotoxic response with rapid and maximal release of LDH and a slow but eventually substantial release of the lysosomal marker, β glucuronidase. The results indicate that a primary cytotoxic effect on the cell can be manifested by rapid and selective result of the cytoplasmic marker enzyme and that under these circumstances release of the lysosomal constituents is delayed but eventually occurs. The results suggest that the response of the neutrophils to the three immunologic reactions is not due to a cytotoxic effect on the cell membrane but rather activation of a specific process that precedes a general increase in cell membrane permeability.

The more rapid release of β glucuronidase as compared to LDH is not explicable on the basis of the size of molecules alone. Most LDH are estimated to have a molecular weight of approximately 140,000 (16) while the molecular weight β glucuronidase from bovine liver has been estimated at 280,000 (17). On the basis of size alone, one would expect LDH to cross the cell membrane more readily.

The present studies do not help to define the actual mechanics of the release process. Granule extrusion or granule fusion with the cell membrane has not been observed with the antibody-coated biopolymer membrane model (10). Henson on the other hand has demonstrated granule extrusion through the invaginated portion of the neutrophil membrane in his non-phagocytic model. This may represent the efforts of the cell to form a phagolysosome. The early selective release of lysosomal material in the phagocytic system is compatible with extrusion of phagolysosomal material, possibly during uptake of new material. Studies have been reported supporting this concept (9, 14). Morphologic studies on the neutrophil anti-neutrophil antibody system are in progress in this laboratory.

Selective release of lysosomal constituents from other cells has also been well documented. Henson noted a selective release of smaller materials from platelet granules in a complement-dependent
nonlytic system in vitro (18), while Holmson and Day reported selective release of β glucuronidase from platelets exposed to thrombin (19). Mast cells will apparently release histamine without undergoing cytolysis in response to both immune reactants (20) and low doses of octylamine (21).

The present experiments indicate that release of at least one lysosomal marker enzyme and perhaps many other bioactive lysosomal factors can occur as a specific response to immunologic stimuli. Furthermore there is no evidence that this response represents a regressive or degenerative cellular phenomenon, although this may ultimately occur. In two of the three reactions lysosomal release appeared to follow a stimulus to the cell surface and did not depend on interiorization of immunologic reactants. Whether the release process in all three reactions operates through a single common activation mechanism remains to be determined.

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