PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 952–957 (1974)

Granule Enzymes from Human Leukocytes: Their Effect on HeLa Cells[1] (37931)

S. B. TAUBMAN AND R. B. COGEN
(Introduced by I. H. Lepow)
Departments of Pathology and Periodontology, University of Connecticut Health Center, Farmington, Connecticut 06032

Lysosomal enzymes derived from human or rabbit leukocytes have been shown by several laboratories to be capable of tissue destruction (1–4). The presence of numerous granule hydrolases in human leukocytes has also been demonstrated (5). Specifically, collagenase and elastase, both proteases active at neutral pH, have been shown to be capable of digesting their respective connective tissue substrates *in vitro* (6). However, the effect of leukocyte lysosomal enzymes on cellular function or viability has not previously been studied.

Lundgren *et al.* (7) studied the effects of human peripheral granulocytes on human fibroblast monolayers. The results suggested that granulocytes, either intact or sonicated, were able to disrupt fibroblast monolayers. However, unlike the effect of sensitized lymphocytes which appeared to mediate the death of the cells of the monolayer, the presence of granulocytes seemed merely to result in release of fibroblasts from their growth surface without affecting their viability. Monolayer cell release was determined by visual observation, and fibroblast viability was assessed qualitatively, but not quantitatively, by replating the released cells and determining their capacity to form a new monolayer. These workers suggested that release of monolayer cells might be mediated by lysosomal enzymes. The present studies were specifically designed to determine and quantify the effects of human leukocyte lysosomal enzymes on cells.

HeLa cells were used as target cells, and a lysate of granules obtained from peripheral blood leukocytes served as the effector. The action of this mixture of lysosomal enzymes on the viability and some metabolic functions of the target cells was studied.

Materials and Methods. The lysosomal granules were obtained from the leukocytes of freshly collected, human blood, as described previously (8). The granules were disrupted by six cycles of freeze-thaw, and debris was removed by centrifugation at 48,000*g* for 30 min. Stock cultures of HeLa cells were originally obtained from Microbiological Associates. The cells were grown in Falcon plastic tissue culture flasks in Waymouth's Medium MB752/1 (Microbiological Associates) supplemented with 10% vol/vol fetal calf serum. For use in these experiments, cells were trypsinized (0.25% trypsin in Hank's balanced salt solution), replated into 35 mm × 10 mm plastic tissue culture petri dishes (Falcon), and allowed to grow to confluency. Cells in those experiments requiring radiolabeling with ^{51}Cr were incubated in the presence of 25 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear) per 2 ml medium for 60 min at 37°. This was followed by five washes with Hank's Balanced Salt Solution (HBSS). All experiments were carried out in tripliate cultures in 2 ml medium without added serum unless specified otherwise. The results

[1] This is manuscript No. 49 from the Department of Pathology, University of Connecticut Health Center, Farmington, Connecticut. Supported by Grant AI-10054, National Institute of Allergy and Infectious Diseases and the University of Connecticut Research Foundation. We thank Mr. Jeffery Malnick for expert technical assistance.

tabulated are the average of the three independent determinations.

After incubation under the conditions of the experiment (37°, 3 hr), the medium above the monolayer was removed and transferred to test tubes. The monolayer was then washed twice with 1-ml portions of HBSS, which was added to the previously removed medium. The remaining monolayer cells were recovered by trypsinization (0.25% trypsin in HBSS 10 min, 37°), and these were added to new test tubes. The petri dishes were subsequently washed twice, and the washes were added to the monolayer fraction. ^{51}Cr was determined on 1.00-ml aliquots of each fraction by counting on a γ-counter (Nuclear Chicago, Model 4320). Lactic dehydrogenase (LDH) was determined as described by Bergmeyer *et al.* (9). Released LDH was measured in the medium (culture fluid) after centrifugation of intact cells ($210g \times 10$ min). Total LDH was the sum of the enzyme activities in the monolayer and culture fluid fractions following cell lysis (0.1% Triton X-100, 37°, 15 min). Cellular viability was determined by dye exclusion using trypan blue. This was measured by counting at least 100 cells in each sample after dilution in 0.2% trypan blue in phosphate-buffered isotonic saline. Release of ^{51}Cr from cells was quantified by measuring ^{51}Cr released into the cell culture supernatant fluid ($210g \times 10$ min).

For the experiments measuring radiolabeling with ^{3}H-leucine, the cells were incubated under experimental conditions followed by incubation in the presence of 1.0 μCi of ^{3}H-leucine for 2 hr in 2.0 ml medium containing 10% serum. The proteins and other macromolecules of the cells were then precipitated with cold 5% trichloroacetic acid (TCA) containing nonradioactive leucine. The precipitate was washed three times with cold TCA and dissolved in 1.0 ml Hyamine hydroxide, which was subsequently added to 10 ml Aquasol liquid scintillant. Counting was in a liquid scintillation counter (Nuclear Chicago, Model Mark II). Hyamine hydroxide and Aquasol were both from New England Nuclear Corp.

Results. In confirmation of the results of Lundgren *et al.*, we found that the addition of human leukocyte granule enzymes[2] to monolayers of HeLa cells resulted in an apparent release of the cells from the surface. Qualitatively the cells, or at least a fraction of them, remained viable as shown by replating into new petri dishes. In this experiment,[3] the culture fluid supernatant (containing the cells that had been released) was removed, and the cells it contained washed once with 2 ml medium containing 10% fetal calf serum. Finally, the cells were resuspended in an additional 2 ml medium containing 10% fetal calf serum and replated. Sequential observation of the plates indicated that these cells reattached, replicated on the new petri dishes, and continued to grow. Unfortunately, such an experimental result, because of its qualitative nature, could mask an effect that either is not lethal, or is lethal for only a fraction of cells. The following experiments were carried out in an attempt to assess, in a quantitative manner, the effects of these enzymes on intact cells.

As seen in Table I, the addition of granule enzymes to HeLa cells grown in monolayers resulted in release of the cells as evidenced by the appearance of ^{51}Cr in the culture fluid and loss of cells from the monolayer. This release was approximately proportional to the concentration of lysosomal enzymes added (Table II) and was

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[2] A typical preparation of granule enzyme has the following activities per milligram protein, generally ascribed to granule hydrolases: (a) lysozyme equivalent to 350 μg egg white lysozyme (Worthington Biochemical Corp.); (b) β-glycerophosphatase, equivalent to 84 milliunits wheat germ acid phosphatase (Worthington Biochemical Corp.); (c) peroxidase, equivalent to 1.4 purpurgallin units horseradish peroxidase (Sigma Chemical Corp.); (d) neutral protease, at pH 7.4 and 37°, 5.8 mg denatured hemoglobin is degraded (5% TCA solubility) in 3 hr (starting with 50 mg hemoglobin). Lactic dehydrogenase activity is not detectable by spectrophotometric assay with NADH and pyruvate.

[3] Lysosomal enzymes (33.5 μg/ml) were added, and the plates were incubated at 37° for 24 hr.

TABLE I. Effect of Leukocyte Granule Contents on HeLa Cell Monolayers.

Additions	Concentration	Monolayer: Cells per high power field[a]	Cells in culture fluid: ^{51}Cr released from monolayer[a]
None	—	33	8.8
Normal human serum	10%	35	8.5
Lysosomal enzyme	160 μg/ml[b]	0	92.2
Normal human serum + Lysosomal enzyme	10% + 160 μg/ml	35	9.0

[a] After 3 hr at 37°C.
[b] μg protein/ml.

readily inhibited by the addition of either normal human serum or heated (56°, 30 min) human serum. While ^{51}Cr release from the monolayer was originally thought to be an indication of cell lysis, it is obvious from Table II that the released cells are still sedimentable and, therefore, intact. Lysis of the monolayer cells by the addition of Triton X-100 resulted in total release of the ^{51}Cr label into the supernatant. As was the case with ^{51}Cr release, LDH, a cytoplasmic marker enzyme, was not present in solution following treatment with lysosomal enzymes, but was readily sedimentable by slow speed centrifugation (Table III). This serves to further indicate that the cells, although released from the monolayer, remained intact.

In the experiment seen in Table IV, the ability of cells released from the monolayer to exclude trypan blue, another criterion of cell viability, was determined. Where a large percentage of the cells had been released from the monolayer, as when lysosomal enzymes alone were added, most of the released cells (84%) retained the ability to excluded trypan blue. When the release was small, as in the control dishes, a large percentage of such cells had lost their viability, as evidenced in the first line in Table IV, where only 23% of the cells excluded trypan blue.

A further indication that after treatment with granule enzymes the cells released were still vital was the ability of these cells to

TABLE II. Effect of Leukocyte Granule Contents on ^{51}Cr Release from HeLa Cells.

Additions	Concentration	Cells in culture fluid: ^{51}Cr released from monolayer (% of total)[a]	Culture fluid supernatant: ^{51}Cr released from cells (% of total)[a,b]
None	—	15.3	14.9
Lysosomal enzyme	72.5 μg/ml[c]	97.7	13.5
Lysosomal enzyme	36.3 μg/ml	89.7	16.4
Lysosomal enzyme	18.2 μg/ml	39.8	16.5
Lysosomal enzyme	9.1 μg/ml	36.5	19.8
Normal human serum	10%	9.0	7.2
Normal human serum + Lysosomal enzyme	10% + 36.3 μg/ml	8.8	7.7

[a] After 3 hr at 37°C.
[b] Centrifuged 210g × 10 min.
[c] μg protein/ml.

TABLE III. Effect of Leukocyte Granule Contents on Lactic Dehydrogenase (LDH) Release by HeLa Cells.

Additions	Concentration (μg protein/ml)	Cells in culture fluid: ^{51}Cr released from monolayer (% of total) [a]	LDH released (% of total) [a]
None	—	7.6	3.1
Lysosomal enzyme	75	96.9	7.0
Lysosomal enzyme	37.5	79.9	2.3
Lysosomal enzyme	18.3	39.9	9.3
Lysosomal enzyme	9.2	13.2	0.4
SBTI[b]	10^3	7.0	3.6
Lysosomal enzyme + SBTI	75 10^3	7.9	1.1

[a] After 3 hr at 37°C.

[b] Soybean trypsin inhibitor (SBTI) was added in this case to avoid complication that would arise from the presence of LDH activity in normal human serum.

incorporate an amino acid, ^{3}H-leucine, into protein-like material. Treatment of HeLa cells with lysosomal enzymes for 3 hr at 37° did not significantly impair the ability of the cells to synthesize protein (as determined by incorporation of ^{3}H-leucine into material precipitable by cold TCA) (Table V). To prevent ^{51}Cr interference with ^{3}H determinations, chromium was not present in the petri dishes to which ^{3}H-leucine was added. However, since this experiment was carried out as part of the experiment seen in Table III, it may be assumed that 97% of the cells were released from the monolayer on addition of 75 μg/ml lysosomal protein.

Discussion. In spite of the presence of multiple hydrolases, some of which are known to be active at neutral pH, the only activity demonstrable in the present experiments with respect to an action related to the HeLa cells consisted of a trypsin-like release of the cells from the monolayer with retention of cell vitality, as determined by five independent criteria. Even this effect is completely ameliorated by the presence of 10% normal human serum or a protase inhibitor such as soybean trypsin inhibitor.

In the earlier report of Lundgren *et al.* (7) it was noted that while intact granulocytes as well as sonicated granulocytes were able to simply disrupt the fibroblast mono-

TABLE IV. Effect of Leukocyte Granule Contents on Viability of HeLa Cells (by Trypan Blue Exclusion).

Additions	Concentration	Cells in culture fluid[a]: ^{51}Cr released from monolayer (% of total)	Trypan blue exclusion (%)
None	—	10.9	23
Lysosomal enzyme	75 μg/ml[b]	93.6	84
Normal human serum	10%	22.5	58
Normal human serum + Lysosomal enzyme	10% 75 μg/ml	10.5	58

[a] After 3 hr at 37°C.

[b] μg protein/ml.

TABLE V. Effect of Leukocyte Granule Contents of HeLa Cells: Protein Synthesis as Measured by ^{3}H-Leucine Incorporation.

Additions	Concentrations	^{3}H-Leu incorporation[a] (cpm)
None	—	2290
Lysosomal enzyme	75 μg/ml[b]	2064
Normal human serum	10%	2237
Normal human serum + Lysosomal enzyme	10% 75 μg/ml	2542

[a] After 3 hr at 37°, 1 μCi ^{3}H-Leucine was added, and incubation was continued an additional 2 hr.

[b] μg protein/ml.

layers, phytohemagglutinin-stimulated intact lymphocytes, but not sonicated lymphocytes, had a toxic effect on the monolayer cells. On the basis of circumstantial data, it was felt by these authors that the factor responsible for "plaque formation" originated in the cytoplasmic granules of the leukocytes. Review of the experimental procedures described indicated that 10% human serum, type AB was added to the medium. In the presence of normal serum it is unlikely that the disruption of fibroblast monolayers by granulocytes is due to the same lysosomal constituents reported in the present study. In our experiments, normal human serum (or heated serum) was sufficient to completely inhibit the disruptive effect of the granule contents.

In a recent study, Mosser *et al.* (10) found that treatment of mouse fibroblasts (3T3/4) with extracts of human leukocyte lysosomes increased the susceptibility of the cells to concanavalin A-induced agglutination. This activity was postulated as being at least partially a result of elastase present in the granulocyte lysosomes. Cell surface changes, resulting from interaction of the lysosomal hydrolases with structures on the membranes of the cells, were considered to have resulted in the change in cell agglutinability. Similar changes might be expected to release cells from their growth surface.

In a report on peroxidase-mediated cell cytotoxicity (11), the addition of a peroxide-generating system (glucose plus glucose oxidase), a peroxide-utilizing system (lactoperoxidase or myeloperoxidase), and halide ion (iodide or chloride) to a variety of mammalian cells resulted in cell death as evidenced by ^{51}Cr release and trypan blue uptake. In the present studies, while the granule enzyme mixture contained relatively the same magnitude of myeloperoxidase activity as the maximum amounts added by Edelson and Cohn, neither H_2O_2 nor a peroxide generating system was specifically added. Under these circumstances, only release of the cells from the monolayer was seen.

It has often been alluded to and widely assumed that lysosomal enzymes have a destructive effect on cells and tissues. These assumptions appear to be based on the well demonstrated effects of these enzymes on extracellular structures, and, also circumstantially, on the powerful hydrolytic enzymes contained within the lysosomes. The data from the present *in vitro* studies using HeLa cells and human leukocyte lysosomal enzymes indicate, however, granule enzymes alone are not able to seriously impair cell viability.

Summary. Granule enzymes were obtained from human peripheral blood leukocytes and added to HeLa cells growing as monolayers. The enzymes disrupted the monolayer, releasing the HeLa cells, but the released cells remained vital as determined by five separate, independent criteria.

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Received Oct. 5, 1973. P.S.E.B.M., 1974, Vol. 145.