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Lysosomal Enzyme Secretion and Volume Contraction Induced in Neutrophils by Cytochalasin B, Chemotactic Factor and A23187[1,2] (38831)

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Chemotactic factors induce an increase in the volume of rabbit peritoneal polymorphonuclear leukocytes and this increase in volume is closely correlated with the chemotactic responsiveness of the cell (1). In contrast, chemotactic factors in the presence of the mold product, cytochalasin B cause a decrease in cell volume which is superimposed on the volume decrease caused by cytochalasin B alone (1). Chemotactic factors in the presence of cytochalasin B also induce the secretion of lysosomal enzymes from neutrophils (2, 3) which is greatly augmented over that caused by either cytochalasin B (2, 4) or chemotactic factors alone (4). In addition to these associations between volume contraction and lysosomal enzyme secretion, this paper will show that the divalent cation ionophore, A23187, also induces lysosomal enzyme secretion from and volume contraction of neutrophils. Also, this work explores what if any relationship exists between the extent of neutrophil lysosomal enzyme secretion and the volume contraction induced by the three stimuli, cytochalasin B, cytochalasin B plus chemotactic factor, and A23187.

Materials and Methods. The polymorphonuclear leukocytes (heterophils, neutrophils) used throughout this study were obtained 12 hr after the injection of 0.02% glycogen into the peritoneum of 22 rabbits as previously described (5). The peritoneal exudate so obtained contained 90–95% neutrophils. For all studies involving lysosomal enzyme release the leukocytes were washed once in Hanks' buffer containing 0.01 *M* Tris, (Tris (hydroxymethyl) amino methane) pH 7.3, 1 mg/ml of glucose, and then suspended in the same buffer with 1 mg/ml of crystalline bovine serum albumin. For all studies involving measurement of the change in volume, the cells were centrifuged from the peritoneal exudate and resuspended in their own exudate fluid at a concentration of 3–5 $\times$ 10^7 neutrophils/ml.

The chemotactic factor employed throughout was a butanol extract of an *E. coli* culture filtrate (6). Before use the butanol was evaporated off at room temperature under vacuum and the residue dissolved to the appropriate concentration in Hank's buffer. The cytochalasin B, purchased from Aldrich Chemical Co., Milwaukee, WI, was dissolved in dimethyl sulfoxide at a concentration of 4 mg/ml and stored at $-20°$ until needed. The ionophore, A23187, was obtained from Eli Lilly and Co., Indianapolis, IN through the courtesy of Dr. Robert P. Hamill to whom we express our thanks. It was stored at 4° dissolved in 95% ethanol at a concentration of 10^{-3} *M* and diluted in Hanks' buffer just before use.

Lysosomal enzyme secretion. Washed neutrophils (1×10^7) in Hanks' buffer were placed in 12 $\times$ 75-mm siliconized tubes. Cytochalasin B was added at a final concentration of 5 μg/ml to the appropriate tubes and all tubes were allowed to stand for 10 min at room temperature. At the end of this time chemotactic factor at a final 1:2000 dilution of the original butanol extract was added. In all instances, the final volume was 2 ml and all tubes were run in duplicate. Control tubes, containing neither cytochalasin B nor chemotactic factor were tested in parallel. When the ability of cytochalasin B to induce secretion was to be measured the

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tubes with appropriate controls were incubated at 37° for 1 hr; when cytochalasin B plus chemotactic factor was the stimulus a 10-min incubation at 37° was employed. when A23187 was the stimulus the cells were incubated with either 10^{-5} *M* or 2×10^{-6} *M* ionophore for 5 min at 37°. These incubation times were chosen on the basis of preliminary experiments to give maximum release. At the end of the respective incubation periods the cell suspensions were centrifuged in the cold at 700*g* (3000 rpm) for 5 min. The supernatants were assayed for the cytoplasmic marker enzyme, lactic dehydrogenase (LDH), and the lysosomal granule enzymes, β gluronidase and lysozyme as previously described (5). The total activity of each of the three enzymes was measured in tubes containing 1×10^7 neutrophils in 2 ml of 0.1% Triton X-100 in Hanks' buffer (5). The activity released into the supernatant was recorded in both enzyme units as described in (5), and as percentage of the total enzyme activity in 1×10^7 cells.

Volume change. For all stimuli, 20 μl of a suspension of $3\text{–}5 \times 10^7$ neutrophils in peritoneal exudate was added to a beaker containing 20 ml of Hanks' buffer and equiliberated at 37° for 10 min with stirring. When cytochalasin B or cytochalasin B plus chemotactic factor was the stimulus, cytochalasin B was added at a final concentration of 5 μg/ml and incubated at 37° for 30 min. At the end of that time the mean cell volume was determined with the ZB1 Coulter Electronic Counter kept in an incubator at 37°. Measurements were done at 37° because the decreases in mean cell volume were found to be greater at 37° than at room temperature. At the end of the measurement, 8 ml were transferred to each of two 10-ml beakers and to one of the beakers 40 μ-l of bacterial factor at a final dilution of 1:2000 was added and the mean cell volume determined immediately; an equal volume of buffer was then added to the control beaker and its mean cell volume was read.

When the ionophore was the stimulus, no cytochalasin B was added to the 20 ml of cell suspension, but it was divided into 10-ml aliquots. To one aliquot 100 μl or 20 μl of ionophore, respectively, at a final concentration of either 1×10^{-5} or 2×10^{-6} *M* was added and the mean cell volume determined after 2-min incubation at 37°. To another 10 ml an equal volume of 95% ethanol was added and after 2 min the mean cell volume was measured.

In all instances the decrease in volume was recorded as the percentage of the mean cell volume of the control cells.

Results. We measured the ability of peritoneal polymorphonuclear leukocytes from 22 rabbits to secrete β-glucuronidase and lysosomal enzymes and to contract their volume in response to cytochalasin B, cytochalasin B plus chemotactic factor, and two concentrations, 1×10^{-5} and 2×10^{-6} *M* of the ionophore A23187. The results are summarized in Table I. In the measurements on cells treated with cytochalasin B alone the sample from one rabbit was lost by accident.

The results of Table I confirmed our previous observations that cytochalasin B greatly contracts the cell volume and also gives a small but significant secretion of lysosomal enzymes (1, 2). Table I also shows that a very large enhancement of lysosomal enzyme secretion is induced by adding bacterial chemotactic factor to cytochalasin B-treated cells and that this is accompanied by a small but significant further decrement in the neutrophils' volume (1, 2). The ionophore A23187 released both lysozyme and β glucuronidase although β glucuronidase to a distinctly lesser extent than lysozyme. Goldstein *et al.* had previously reported this for neutrophils of human peripheral blood (7). Of interest was the finding (Table I) that 10^{-5} *M* ionophore induced distinctly more leakage of LDH and significantly less lysosomal enzyme release than did 2×10^{-6} *M* A23187. Whether these effects of the higher concentration of ionophore were due to the interaction of the 1% ethanol and the ionophore is not known. The ionophore also induced a decided decrease in neutrophil volume; there being little difference between the over-all degree of contraction induced by the two concentrations.

As far as comparisons between different stimuli are concerned, no relation is apparent between the average lysosomal enzyme secretion and the average degree of reduction in

TABLE I. RELATION OF THE MEAN PERCENT LYSOSOMAL ENZYME RELEASE TO PERCENT VOLUME DECREASE INDUCED BY THREE DIFFERENT STIMULI.

	Cytochalasin B	Cytochalasin B[c] plus bacterial factor	A21387		Total enzyme activity (units/2 ml)
			10^{-5} M	2×10^{-6} M	
Lysozyme	6.1 ± 0.66[a]	55 ± 1.6	8.8 ± 1.4	17 ± 1.4	410 ± 28
β glucuronidase	1.9 ± 0.23	30 ± 1.7	3.4 ± 0.59	6.3 ± 0.57	15.0 ± 0.74
LDH	0.26 ± 0.22	0.24 ± 0.12	1.73 ± 0.19	0.45 ± 0.13	1484 ± 100
Volume decrease	16.9 ± 1.49[b]	3.37 ± 0.61	15.4 ± 1.46	16.9 ± 1.1	
Total number of cell samples tested	21	22	22	22	

[a] All results of enzyme release except those in last column are reported as percentage of total enzyme.
[b] Volume decrease is reported as the percentage of the initial volume measured before addition of stimulus.
[c] The volume decrease is the decrement caused by the addition of bacterial factor to cells already contracted by the prior addition of cytochalasin B.

TABLE II. CORRELATION BETWEEN ENZYME RELEASE AND VOLUME DECREASE OF POLYMORPHONUCLEAR LEUKOCYTES STIMULATED BY CYTOCHALASIN B, CYTOCHALASIN PLUS CHEMOTACTIC FACTOR, AND THE IONOPHORE.

Stimulus	Correlation coefficient of percentage enzyme release versus percent contraction			N[a]
	LDH	Lysozyme	β Glucuronidase	
Cytochalasin B	.089	.278	.031	21[b]
Cytochalasin B plus chemotactic factor	−.012	−.336	−.142	22
A23187				
1×10^{-5} M	.038	−.29	−.486*	22
2×10^{-6} M	.206	.081	.155	22

* Statistically significant, $P < 0.05$.
[a] N = number of leukocyte samples tested.
[b] One sample lost by accident.

cell volume. Cytochalasin B by itself, induced an average decrease in cell volume which was about five times the incremental decrease caused by the addition of bacterial factor to cells already contracted by the prior addition of cytochalasin B, yet the lysosomal enzyme release induced by cytochalasin B alone was one tenth or less of that caused by chemotactic factor plus cytochalasin B. The average decrease of neutrophil volume induced by 2×10^{-6} M A23187 is the same as that induced by cytochalasin B but the lysosomal enzyme release was approximately one half or less than that caused by the latter stimulus.

Although no relationship could be detected between the average lysosomal enzyme release and volume contraction when different stimuli were compared it was possible that for a given stimulus some sort of relationship between these two variables did exist. To test this possibility the linear correlation coefficients of the degree of contraction versus the percentage release of each of the three enzymes as measured on the 22 different cell samples was calculated for each stimulus. The results of these calculations are given in Table II. With only one exception, for no stimulus was any statistically significant correlation found between the degree of release of any enzyme and the extent of contraction of the volume. In the single exception the correlation between β glucuronidase release and volume contraction caused by 1×10^{-5} M A23187, the correlation coefficient was −0.49, that is, there was a tendency for cells giving higher β glucuronidase release to give lower degrees of contraction. The meaning of this negative correlation is as unclear as its reality is uncertain.

At the 5% level of significance, one would expect one out of 20 statistically significant correlations to arise by chance alone; here one finds one out of 12.

No essential difference was found if the correlation coefficients were recalculated using instead of percentage enzyme release the absolute enzyme units.

Discussion. To the extent studied, there is a clear-cut association between induced lysosomal enzyme release from and contraction of the cell volume of polymorphonuclear leukocytes. The previous brief report demonstrating this for polymorphonuclear leukocytes induced to secrete by cytochalasin B and by chemotactic factors in the presence of cytochalasin B is fully confirmed here (Table I). This is also true when the divalent cation ionophore A23187 is the stimulant (Table I). Recently, we have found that neutrophils suspended in a low-ionic-strength glucose medium secrete lysosomal enzymes when stimulated by cytochalasin B and ATP[3] and in unpublished work have demonstrated an associated decrease in volume.

The association between the decrease in cell volume and lysosomal enzyme release caused by these very diverse stimuli suggests that the decrease in cell volume may be part of the process of lysosomal enzyme secretion. The lack of correlation between the magnitude of the secretion and the decrease in volume indicates, however, that the volume decrease can not be a direct cause of the lysosomal enzyme secretion, for example, a given stimulus inducing an activation of the contractile mechanism of the cell causing a reduction in cell volume and a squeezing of the lysosomal granules toward the cell periphery. Similarly, the lack of correlation precludes the decrease of cell volume being a direct result of the loss of granule contents with a consequent loss of water. One remaining possibility is that the various stimuli induce an efflux of cation(s) from the cell with a consequent loss of water and decrease in cell volume. This putative cation efflux can be part of or an epiphenomenon unrelated to the process of lysosomal enzyme secretion. Gallin and Rosenthal have shown that chemotactic factors induce a net efflux of intracellular Ca^{2+} but that this is reduced in the presence of cytochalasin B (8). This is opposite of what is expected on the basis of the volume changes reported here and thus cannot be their cause. Dunham *et al.* have recently shown that phagocytic stimuli in the presence or absence of cytochalasin B cause a decrease in activity of the K^+ pump of human neutrophils (9). This suggests that the volume decrease associated with induced lysosomal enzyme secretion reported here might be caused by a net efflux of K^+.

Summary. The secretion of lysosomal enzymes from rabbit peritoneal polymorphonuclear leukocytes caused by either cytochalasin B, or cytochalasin B plus chemotactic factors, or by the divalent ionophore, A23187, is associated with a decrease in the volume of these same cells. No quantitative correlation could be found between the extent of lysosomal enzyme secretion and volume decrease for any of the three stimuli. This suggests that the volume decrease is neither the direct cause nor the result of the secretion.

[3] Elmer L. Becker, and Peter Henson. Manuscript submitted.

1. Hsu, L. S., and Becker, E. L., Fed. Proc. **33,** 603 (1974).
2. Becker, E. L., and Showell, H. J., J. Immunol. **112,** 2055 (1974).
3. Goldstein, I. M., Hoffstein, S., Gallin, J., and Weissman, G., Proc. Nat. Acad. Sci. USA **70,** 844 (1973).
4. Davies, P., Fox, R. I., Polyzonis, M., Allison, A. C., and Haswell, D. A., Lab Invest. **28,** 16 (1973).
5. Becker, E. L., Showell, H. J., Henson, P. M., and Hsu, L. S., J. Immunol. **112,** 2047 (1974).
6. Schiffman, E., Showell, H. J., Corcoran, B., Smith, E., Ward, P. A., and Becker, E. L., Fed. Proc. **33,** 631 (1974).
7. Goldstein, I. M., Horn, J. D., Kaplan, H. B., and Weissmann, G., Biochem. Biophys. Res. Commun. **60,** 807 (1974).
8. Gallin, J. I., and Rosenthal, A. S., J. Cell Biol. **62,** 594 (1974).
9. Dunham, P., Goldstein, I. M., and Weissmann, G., J. Cell Biol. **63,** 215 (1974).

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