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Release of Marker Lysosomal Enzymes by 3-Methylindole and Indole from Rabbit Lung Lavage Cells (39652)[1]

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Introduction. 3-Methylindole (3MI) is a metabolite produced during *in vitro* incubation of tryptophan, indoleacetic acid, and other indolic compounds with ruminal fluid (1) and, in cattle, after intraruminal doses of L-tryptophan (2). 3-Methylindole has been shown to cause acute pulmonary edema and interstitial emphysema in cattle and goats (3, 4). Clinical signs of pulmonary disease appear within one to several hours after administration, and these animals often die within a few hours to days depending upon the dose and method of administration (3, 4). Pulmonary edema is a predominant early change in the development of pulmonary lesions.

Indole and 3MI can rupture biological membranes, and these effects may be related to the lipophilic properties of these indoles. Indole and 3MI cause cell immobility and disintegration of the membranous structure of ciliated ruminal protozoa (5). These and other aryl compounds hemolyze erythrocytes (6) and cause lesions in rabbit knee joints which suggest membrane damage (7, 8). High concentrations of 3MI intercalate between fatty acid chains of lecithin micelles near the polar head which alters micelle structure (9) and induces structural perturbations in lipid and protein regions of erythrocyte membranes (10, 11).

Proteases from leukocytes, alveolar macrophages, and other sources have been shown to cause pulmonary lesions (12–16). Since lysosomes are intracellular and the enzymes are contained by lysosomal membranes, this study was conducted to determine (a) whether 3MI and indole would cause the release of marker lysosomal enzymes from rabbit lung lavage cells (LLC) *in vitro*, (b) and whether specific chemical agents would modify the effects of 3MI on LLC.

Materials and methods. Experiment 1. New Zealand white rabbits of both sexes, weighing about 2.5 kg, were used to harvest LLC by the method of Myrvik *et al.* (17) as modified by Tsan and Berlin (18). These techniques have been shown to yield at least 90% alveolar macrophages and small numbers of polymorphonuclear cells and lymphocytes (17, 18). Cold modified Hanks' solution (MHS) was used for washing out free lung cells and, for cell suspensions (approximately 70×10^6 cells were obtained from each rabbit). Cells were counted with a hemocytometer, and a suspension of 20×10^6 cells per ml was prepared.

One milliliter of a cell mixture containing 4×10^6 cells and 0, 200, 300, and 400 ppm (0, 1.53, 2.29, and 3.05 m*M*) of 3MI[3] or 0, 400, 600, and 800 ppm (0, 3.42, 5.13, and 6.84 m*M*) of indole[3] were incubated for 0, 1, 2.5, and 4 hr at 37° in a shaking water bath. The cell suspensions were then centrifuged for 10 min at 1200*g*. Two marker lysosomal enzymes, β-glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.31) and β-galactosidase (β-D-galactoside glucuronohydrolase, EC 3.2.1.23) were assayed in the supernatant by the method of Yarborough *et al.* (19). Soluble enzyme activity after incubation was expressed as a percentage of total enzyme activity released by sonication of unincubated cell suspensions.

Experiment 2. The effect of chemical agents[4] on the 3MI-induced release of β-galactosidase from LLC was tested using acetylcholine, acetylsalicylic acid, brain ex-

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tract, cortisone, dimethylsulfoxide (DMSO), L-epinephrine, lysolecithin, phosphatidyl serine, retinoic acid, sphingomyelin, D-α-tocopherol, and Triton X-100. Selected amounts of acetylsalicylic acid, cortisone, retinoic acid, and D-α-tocopherol were dissolved in DMSO and then mixed with MHS so that a final concentration of 5% DMSO was used in all incubation mixtures with these compounds. The remaining compounds were dissolved in MHS. Sonication was used to make emulsions of brain extract, lysolecithin, phosphatidyl serine, and sphingomyelin. The final incubation mixtures were made by mixing 0.2 ml of LLC suspension (4×10^6 cells), 0.8 ml of 500 ppm of 3MI [(363 ppm) (2.77 m*M*) final concentration], and 0.1 ml of each agent. Mixtures were incubated at 37° in a shaking bath for 2.5 hr. β-Galactosidase activity was measured, and the soluble enzyme activity in the supernatant was expressed as in Expt 1. The enzyme activity released from cells in a control tube, without 3MI and chemical agents, was subtracted to give the net enzyme release. The effect of DMSO on the activity of solubilized β-galactosidase was tested in sonicated cell suspensions with or without 5% DMSO. The data were analyzed using Duncan's new multiple range test and Student's *t*-test for significant differences due to treatment for Expts 1 and 2, respectively.

Results. Experiment 1. The total activities of β-galactosidase and β-glucuronidase released by sonication from LLC were 589 ± 9 and 38 ± 2 nmole/hr/4 × 10^6 cells, respectively. Incubation of LLC with increasing concentrations of 3MI and for longer times significantly increased the activities of β-galactosidase and β-glucuronidase in the supernatant (Table I). Soluble β-galactosidase activity was significantly higher than the control at 4 hr in 200 ppm of 3MI, at 2.5 and 4 hr in 300 ppm, and at all incubation times in 400 ppm. Soluble β-galactosidase activity increased almost linearly with time in 300 ppm of 3MI. About 40 and 58% of the total β-galactosidase activity was solubilized in 400 ppm of 3MI at 1 and 4 hr, respectively. After a 4-hr incubation, the soluble enzyme activities at each 3MI concentration were significantly different from each other. Control incubations did not result in a significant increase in soluble enzyme activity with time.

Similar results were obtained with β-glucuronidase except that slightly less activity was released at 400 ppm. Soluble β-glucuronidase activity was significantly higher than the control after 1, 2.5, and 4 hr of incubation with 300 and 400 ppm of 3MI. After 4 hr, the β-glucuronidase activities were significantly different from each other at all concentrations of 3MI.

The activities of β-galactosidase and β-glucuronidase also increased in the supernatant after incubation of LLC with indole, but more than two times the concentration of indole was required for equivalent release compared to 3MI (Table II). Soluble β-galactosidase activities were significantly

TABLE 1. RELEASE OF β-GALACTOSIDASE (β-GAL) AND β-GLUCURONIDASE (β-GLU) ACTIVITY FROM LUNG LAVAGE CELLS BY INCUBATION WITH 3MI.

Incubation time (hr)	Enzyme	Percentage of total enzyme activity at 3MI concentration (ppm) 0	200	300	400
0	β-Gal	2.0 ± 1.4[a]			
	β-Glu	1.9 ± 1.9			
1	β-Gal	3.3 ± 1.6	6.1 ± 1.7	11.7 ± 2.3	39.9 ± 4.5
	β-Glu	3.1 ± 0.7	6.5 ± 3.8	12.2 ± 1.4	25.5 ± 3.2
2.5	β-Gal	3.7 ± 1.6	9.1 ± 0.9	21.2 ± 3.2	47.3 ± 6.1
	β-Glu	4.1 ± 2.7	14.3 ± 5.6	21.9 ± 7.1	41.7 ± 2.1
4	β-Gal	5.5 ± 1.8	17.5 ± 4.7	32.2 ± 0.2	57.7 ± 3.5
	β-Glu	3.6 ± 2.5	16.0 ± 5.6	30.5 ± 3.1	46.0 ± 4.0

[a] Each value is the mean ± SEM of three experiments. Values not sharing the same line are significantly different from each other ($P < .05$).

TABLE II. RELEASE OF β-GALACTOSIDASE (β-GAL) AND β-GLUCURONIDASE (β-GLU) ACTIVITIES FROM LUNG LAVAGE CELLS BY INCUBATION WITH INDOLE.

Incubation time (hr)	Enzyme	Percentage of total enzyme activity: Indole concentration (ppm) 0	400	600	800
0	β-Gal	2.8 ± 1.0[a]			
	β-Glu	2.2 ± 2.2			
1	β-Gal	2.3 ± 1.2	6.3 ± 1.2	10.2 ± 0.6	24.8 ± 8.9
	β-Glu	2.5 ± 0.9	8.7 ± 2.9	12.1 ± 3.4	18.7 ± 5.2
2.5	β-Gal	3.7 ± 1.2	7.8 ± 1.3	18.1 ± 2.0	34.3 ± 6.6
	β-Glu	4.6 ± 2.4	11.8 ± 4.7	18.4 ± 6.0	25.5 ± 6.9
4	β-Gal	4.8 ± 1.9	10.7 ± 2.1	24.1 ± 2.9	38.8 ± 5.7
	β-Glu	4.2 ± 2.1	19.6 ± 5.2	26.3 ± 4.5	32.1 ± 6.2

[a] Each value is the mean ± SEM of three experiments. Values not sharing the same line are significantly different from each other ($P < 0.05$).

higher than the control at 2.5 hr with 600 ppm and at all incubation times with 800 ppm of indole. After a 4-hr incubation, 4.8, 10.7, 24.1, and 38.8% of the activity was solubilized at 0, 400, 600, and 800 ppm of indole, respectively. The same trends were evident for the release of β-glucuronidase activity by indole.

Experiment 2. The effect of freezing and thawing cell suspensions in the presence and absence of indole was tested, and the β-galactosidase activity released was compared to sonication. Enzyme activity released by Triton X-100 (1000 ppm) was comparable to that released by sonication. Freezing and thawing the LLC (two times) released 44% of the total β-galactosidase released by sonication, and indole (400 ppm for 4 hr) released only 15%. Freezing and thawing in the presence of indole resulted in 81% soluble activity, indicating that indole increased the effectiveness of freezing and thawing in releasing this lysosomal enzyme. Indole had no effect on enzyme activity release by sonication.

The effects of 12 agents on the release of β-galactosidase activity from LLC in the presence and absence of 3MI are shown in Tables III and IV. The incubation conditions of 2.5 hr and 363 ppm of 3MI and concentrations of chemical agents were selected to produce moderate enzyme release.

Low concentrations of Triton X-100, phosphatidyl serine, and lysolecithin caused significant enzyme activity release when incubated alone (Table III). In the presence of 3MI, Triton X-100 and phosphatidyl serine significantly increased soluble enzyme activity above that released by 3MI alone. Lysolecithin, in combination with 3MI, resulted in significantly more enzyme activity release than 3MI alone, but significantly less

TABLE III. MODIFICATION OF 3MI EFFECTS ON LUNG LAVAGE CELLS BY CHEMICAL AGENTS.

Treatment and concentration of agent[a]	Enzyme release[b] (Percentage of total activity): Without 3MI	With 3MI
Control	0[a]	20.1 ± 0.6[f]
Acetylcholine (18.2 ppm)	3.6 ± 0.4[a]	17.4 ± 0.9[fi]
Brain extract (400 ppm)	4.0 ± 0.9[a]	21.2 ± 2.4[fi]
L-Epinephrine (18.3 ppm)	1.0 ± 2.4[a]	20.1 ± 0.9[f]
Lysolecithin (400 ppm)	102.8 ± 4.4[b]	77.5 ± 5.7[g]
Lysolecithin (100 ppm)	67.1 ± 6.8[c]	52.7 ± 0.2[h]
Phosphatidyl serine (400 ppm)	8.9 ± 1.1[d]	33.2 ± 3.7[i]
Sphingomyelin (400 ppm)	6.7 ± 2.0[ad]	15.0 ± 1.5[j]
Triton X-100 (100 ppm)	22.4 ± 3.2[e]	67.2 ± 2.9[k]

[a] Four million LLC were incubated for 2.5 hr at 37° with or without 363 ppm (2.77 m*M*) of 3MI and chemical agents. Small amounts of enzyme release in control incubations were subtracted from all values to give net activity released. Each value is the mean ± SEM of three experiments.

[b] Means in each column with different superscripts are significantly different ($P < 0.05$).

TABLE IV. MODIFICATION OF 3MI EFFECTS ON LUNG LAVAGE CELLS BY CHEMICAL AGENTS DISSOLVED IN DMSO.

Treatment and concentration of agent[a]	Enzyme release[b] (percentage of total activity)	
	Without 3MI	With 3MI
Control	0[a]	20.1 ± 0.6[c]
DMSO (5%)	−1.2 ± 0.4[a]	7.4 ± 0.3[d]
Acetylsalicylic acid (60 ppm) + DMSO	0.1 ± 0.5[a]	47.4 ± 2.3[e]
Retinoic acid (10 ppm) + DMSO	4.8 ± 1.2[b]	14.2 ± 1.9[f]
D-α-Tocopherol (1.1 ppm) + DMSO	0.2 ± 0.8[a]	6.2 ± 2.3[d]
Cortisone (360 ppm) + DMSO	1.9 ± 1.4[ab]	10.9 + 3.5[cdf]

[a] Four million LLC were incubated for 2.5 hr at 37° with or without 363 ppm (2.77 m*M*) of 3MI and chemical agents. Small amounts of enzyme release in control incubations were subtracted from all values to give net activity released. Each value is the mean ± SEM of three experiments.

[b] Means in each column with different superscripts are significantly different ($P < 0.05$).

than lysolecithin alone at the concentrations used. With the exception of sphingomyelin which significantly reduced the effect of 3MI, the remaining compounds tested did not alter the effects of 3MI at the concentrations used.

Compared to the control without 3MI, only retinoic acid caused a small but significant enzyme release (Table IV). Dimethylsulfoxide significantly reduced enzyme release in the presence of 3MI from 20.1 to 7.4%. In the presence of 3MI, acetylsalicylic acid significantly increased soluble enzyme activity to 47.4% which was greater than DMSO, 3MI control, or acetylsalicylic acid alone. Retinoic acid significantly increased the soluble enzyme activity compared to the DMSO-3MI control (14.2 vs 7.4%). Dimethylsulfoxide (5%) had no effect on the activity of β-galactosidase in the assay system.

Discussion. The results of this study demonstrate that 3MI and indole can cause the release of β-galactosidase and β-glucuronidase from LLC. Whole cells were used, indicating that these indoles affect both cellular and lysosomal membranes in causing the release of the marker enzymes. The amount of enzyme activity solubilized is associated with the concentration of indole compounds and time of incubation. The time course and amount of β-galactosidase and β-glucuronidase released from LLC were similar during incubation with 3MI and with indole. Compared to 3MI, approximately two times the concentration of indole was required to give equivalent enzyme release. This suggests that the lipophilic properties of these indoles are important since the aqueous solubility of indole is approximately two times that of 3MI and it is less lipophilic than 3MI (6).

The data suggest that 3MI and indole cause enzyme release by rupturing cellular and lysosomal membranes. Release of β-galactosidase by 400 ppm of 3MI and 800 ppm of indole for 4 hr approximated the release by freezing and thawing the cell suspensions two times. β-Galactosidase and β-glucuronidase activities were released in similar relative amounts and in a similar time course. Indole nearly doubled the release of β-galactosidase from LLC subjected to freezing and thawing. This suggests that indole may enhance rupture of the LLC during freezing and thawing and/or promote the release of this enzyme activity from lysed cellular or lysosomal fragments.

The data in Table IV indicate that acetylsalicylic acid, Triton X-100, and phosphatidyl serine enhance the release of the lysosomal enzymes from LLC by 3MI, and that 3MI decreases the release caused by lysolecithin. Even though varying the concentrations of each chemical agent may affect the degree of enzyme release, the results indicate that some compounds enhance the release of lysosomal enzyme activity in the presence of 3MI and suggest that LLC may be more susceptible to lysis under these conditions.

The protective effect of DMSO was unexpected since it had been previously used as a carrier for water-insoluble compounds, and it was reported not to affect lysosomal stability or modify the effects of drugs on lysosomes (20).

The manner by which 3MI decreased the membrane-labilizing effect of lysolecithin is not known at this time. 3MI has been shown to bind with lecithin micelles *in vitro* (9) and may have interacted directly with lysoleci-

thin in the solution and reduced its effect on LLC.

Summary. Rabbit lung lavage cells (LLC) were incubated with 0, 200, 300, and 400 ppm (0, 1.53, 2.29, and 3.05 m*M*) of 3-methylindole (skatole, 3MI) or 0, 400, 600, and 800 ppm (0, 3.42, 5.13, and 6.84 m*M*) of indole for up to 4 hr at 37°. The release of β-galactosidase and β-glucuronidase activities was compared to the total enzyme activity solubilized by sonication. Significant increases in soluble enzyme activity were obtained with these concentrations of 3MI, and the amount of enzyme activity solubilized depended upon the 3MI concentration and incubation time. After incubation with 400 ppm of 3MI for 4 hr, 58% of the total β-galactosidase and 46% of the β-glucuronidase activities was solubilized from LLC. This activity was greater than that released by freezing and thawing two times. At higher concentrations, indole caused a similar release of these lysosomal enzyme activities. Significantly more β-galactosidase activity was released when LLC were incubated with 3MI in combination with low concentrations of acetylsalicylic acid, retinoic acid, Triton X-100, and phosphatidyl serine than by incubation with each agent alone. Incubation with DMSO decreased enzyme release by 3MI, and 3MI inhibited some of the membrane-labilizing effects of lysolecithin. The data indicate that 3MI and indole can cause the release of marker lysosomal enzymes from LLC and that the effects of 3MI can be enhanced or decreased by the presence of some other lipotrophic agents.

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