Effects of Compounds which Inhibit Antigenic Release of Histamine and Phagocytic Release of Lysosomal Enzyme on Glucose Utilization by Leukocytes in Humans[1] (34559)

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Antigenic release of histamine from tissues or leukocytes of sensitized subjects has been shown to be inhibited by nicotinamide (1), ethanol (2), theophylline, and cyclic 3′,5′-adenosine monophosphate (3). In the present study the action of these particular compounds on phagocytic release of a lysosomal enzyme, β-glucuronidase, from leukocytes was also determined and found to be inhibitory. As such cellular responses require expenditure of energy, and glucose is a prime source of energy in leukocytes (4), experiments were conducted to ascertain whether the compounds under scrutiny affected glucose utilization by leukocytes. The compounds had suppressive effects on glucose utilization corresponding to their inhibitory actions on release of histamine and β-glucuronidase (5).

Materials and Methods. Dextran, average molecular weight 280,000, Pharmachem. Heparin, 1000 units/ml, USP. Tris (hydroxymethyl) aminomethane (Trizma, pH 7.6), Sigma. Antigens: ragweed and alternaria aqueous extracts, Center Laboratories, and cows' milk proteins (Pentex). Zymosan (kindness of Dr. Peter Elsbach). Dibutyryl cyclic 3′, 5′-adenosine monophosphate sodium (cyclic AMP), Schwarz Bioresearch. Theophylline HBr, Mann; nicotinamide, Mann; D-glucose-U-^{14}C, Calbiochem; Triton X-100, Rohm and Haas.

The experiments were carried out with leukocytes separated from the blood of normal and allergic persons. Each 10 ml blood was mixed with 2 ml 3% dextran in 0.9% NaCl solution and allowed to settle at room temperature. The leukocyte-plasma layer was separated from the erythrocyte layer and centrifuged at 170*g* for 10 min. The leukocytes were resuspended in a solution of composition and volume required by the methods to be described.

For antigenic histamine release, a procedure similar to that of Osler and Lichtenstein (6), modified to reduce amount of blood required, was employed. Antigen was incubated with leukocytes of hypersensitive persons suspended in a Tris buffer containing Ca^{2+} and Mg^{2+} (Tris-CM, pH 7.6). The leukocytes obtained by centrifugation were washed twice with 3 ml Tris buffer containing 0.03 % human albumin and finally resuspended in Tris-CM buffer equal in volume to the original whole blood-dextran mixture. This usually gave about 1.5 to 2 $\times$ 10^6 mixed leukocytes per ml. A mixture of the leukocyte suspension and antigen was incubated at 37° for 1 hr. Fluorometric analyses of the histamine content of the supernate from the incubation mixture and of an aliquot of the untreated leukocyte suspension permitted calculation of the percentage histamine released from the leukocytes.

Release of histamine and β-glucuronidase during phagocytosis was determined with suspensions of leukocytes in Tris-CM incubated with zymosan particles (10 to 20/leucocyte) for 1 hr. Percentage histamine release was ascertained as described for antigenic release. β-glucuronidase activity in aliquots of the cell suspension treated with 0.2 % Triton X-100 (for the "total") and in

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TABLE I. Release of Histamine and Lysosomal Enzyme during Incubation of Sensitive Leukocytes with Antigen.

Exp.	Antigen	Conc. (μg pN/ml)[a]	Net release (%)	
			Histamine	β-Glucuronidase
128-B	Ragweed	.6	83	1.4
213-O	Alternaria	.0001	71	1.7
128-D	Ragweed	.06	15	0
128-D	Ragweed	.2	38	0
128-D	Ragweed	.6	64	0

[a] μg pN/ml = μg protein nitrogen/ml.

the supernate of aliquots incubated with zymosan (for "released"), from which percent release was calculated, was measured with phenolphthalein-glucuronate as substrate (7). Where in the Tables data are given for release of both histamine and β-glucuronidase, these were determined simultaneously with aliquots of the same leukocyte suspension from a single person for each experiment.

Conversion of glucose to CO_2 was determined by incubation of a suspension of leukocytes with glucose-U-^{14}C and absorbing $^{14}CO_2$ evolved in a closed vessel, as described in the literature (8). Blood for glucose studies was collected and leukocytes separated as already described, except that no glucose was added to the dextran solution. Leukocytes obtained by centrifugation of the plasma layer were not washed, the plasma being decanted and the cell button used directly to prepare the leucocyte suspension in Tris-CM (0.5 ml for each 1 ml whole blood-dextran mixture). The incubation mixture included: 0.5 ml leukocyte suspension; 0.5 ml D-glucose-U-^{14}C (1 μCi/ml); and compounds under study were added in 50 to 100 μl Tris-CM to achieve concentrations shown in the Tables. Radioactivity from CO_2 evolved was measured in a liquid scintillation spectrophotometer. Results were expressed as cpm from $^{14}CO_2$ evolved during incubation.

Results. In Tables I and II it may be seen that (a) antigenic release of histamine from sensitive leukocytes is not accompanied by extrusion of the lysosomal enzyme β-glucuronidase, and (b) the extrusion of β-glucuronidase from leukocytes during phagocytosis of zymosan particles occurs without simultaneous release of histamine. These findings were consistently confirmed in 10 experiments with leukocytes from three subjects and three different antigens. Table III and Fig. 1 show inhibition of histamine release was proportional to concentrations of the compounds and the rank order of potencies was cyclic AMP, theophylline, nicotinamide, and ethanol.

TABLE II. Release of Lysosomal Enzyme and Histamine from Leukocytes during Phagocytosis of Zymosan Particles.

Exp.	Net release (%)	
	Histamine	β-Glucuronidase
128-D	0	24.4
128-E	1	23.2
128-L	2	29.0

The degree of inhibition of extrusion of β-glucuronidase accompanying phagocytosis by these compounds was not consistently dependent on their concentrations; straight-line relationship of concentration-response could not be obtained. The data in Table IV give the maximum inhibitions observed.

Data on the inhibitory effects of the compounds under consideration on glucose utilization by leukocytes, as measured by conversion to CO_2, are presented in Table V and shown graphically in Fig. 1 Inhibition of utilization of glucose was proportional to concentrations of the compounds; the magnitudes of inhibitory concentrations for histamine release and glucose utilization were

TABLE III. Chemical Inhibition of Antigenic Release of Histamine from Sensitive Leukocytes.

Exp.	Antigen	Conc.	Chemical	Conc. (M)	Release of histamine: Net (%)	Release of histamine: vs. control (%)
294-I	Milk proteins	2.0 mg/ml	Control		31	
	Milk proteins	2.0 mg/ml	Cyclic AMP	1×10^{-4}	25	—19
	Milk proteins	2.0 mg/ml	Cyclic AMP	5×10^{-4}	13	—58
	Milk proteins	2.0 mg/ml	Cyclic AMP	1×10^{-3}	7	—78
253-K	Ragweed	.2 μg pN/ml	Control		35	
	Ragweed	.2 μg pN/ml	Theophylline	1×10^{-4}	30	—14
	Ragweed	.2 μg pN/ml	Theophylline	5×10^{-4}	13	—63
	Ragweed	.2 μg pN/ml	Theophylline	1×10^{-3}	4	—88
128-M	Ragweed	.6 μg pN/ml	Control		75	
	Ragweed	.6 μg pN/ml	Nicotinamide	1×10^{-2}	54	—28
	Ragweed	.6 μg pN/ml	Nicotinamide	2×10^{-2}	28	—63
	Ragweed	.6 μg pN/ml	Nicotinamide	4×10^{-2}	9	—88
128-GG	Ragweed	.6 μg pN/ml	Control		71	
	Ragweed	.6 μg pN/ml	Ethanol	5×10^{-2}	60	—15
	Ragweed	.6 μg pN/ml	Ethanol	1×10^{-1}	47	—38
	Ragweed	.6 μg pN/ml	Ethanol	1.5×10^{-1}	31	—56

strikingly similar as was the rank order of potencies for the two processes.

The compounds under consideration did not appear to have deleterious gross effects on the leukocytes, in the concentrations and periods of incubation used in the experiments, as judged by the following: (a) Exclusion of the vital dye eosin-Y was essentially the same as by untreated leukocytes, the dye entering only 1–2% of cells in 1-hr incubation with the highest concentration of the compounds employed, and (b) histamine did

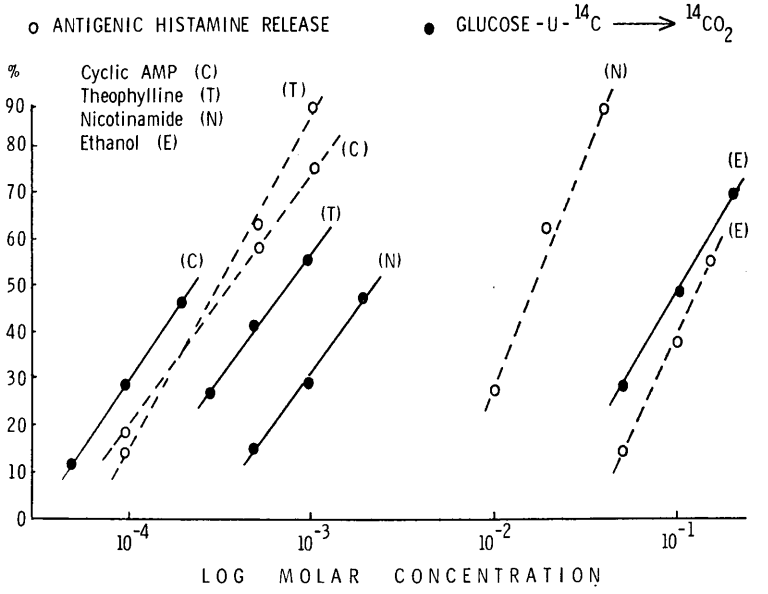

FIG. 1. Inhibition of glucose utilization and histamine release.

TABLE IV. Chemical Inhibition of Release of a Lysosomal Enzyme during Phagocytosis of Zymosan Particles.

Exp.	Agents	Conc. (*M*)	Release of β-glucuronidase	
			Net (%)	vs. control (%)
128-T	Zymosan control		26.1	
	Zymosan + cyclic AMP	1×10^{-2}	15.4	—40
	Zymosan + theophylline	1×10^{-3}	11.2	—57
	Zymosan + nicotinamide	1×10^{-2}	16.1	—38
	Zymosan + cyclic AMP + theophylline	1×10^{-2} + 1×10^{-3}	5.9	—77
XI-55	Zymosan control		31.7	
	Zymosan + ethanol	4×10^{-1}	26.9	—16

not leak from leukocytes incubated with the compounds unless specific antigen was added, nor did the compounds alone in absence of phagocytosis cause extrusion of β-glucuronidase. Free zymosan particles were stained pink by eosin-Y, but phagocytized particles within intact cells are not reached by this stain and so remained colorless. Judged by this means, phagocytic ingestions of zymosan particles seemed to be abundant (three or more particles in over 50 % of leucocytes) and did not appear significantly curtailed in the presence of each of the compounds under the conditions in the present study.

When sensitive leukocytes were incubated with the compounds for 10 min and then centrifuged and washed with Tris-saline buffer before adding specific antigen to re-

TABLE V. Chemical Inhibition of Glucose Utilization by Leukocytes, as Revealed by Conversion of Glucose-U-^{14}C to $^{14}CO_2$.

Exp.	Chemical	Conc. (*M*)	$^{14}CO_2$ formed	
			(Net cpm)	vs. control (%)
X-119	Blank		39	
	Leukocyte suspension (control)		1773	
	Cyclic AMP	5×10^{-5}	1560	—12
	Cyclic AMP	1×10^{-4}	1276	—29
	Cyclic AMP	2×10^{-4}	944	—47
X- 89	Blank		36	
	Leukocyte suspension (control)		1623	
	Theophylline	3×10^{-4}	1167	—28
	Theophylline	5×10^{-4}	909	—44
	Theophylline	1×10^{-3}	716	—56
X- 75	Blank		38	
	Leukocyte suspension (control)		2806	
	Nicotinamide	5×10^{-4}	2360	—16
	Nicotinamide	1×10^{-3}	1967	—30
	Nicotinamide	2×10^{-3}	1447	—48
X- 81	Blank		40	
	Leukocyte suspension (control)		3743	
	Ethanol	5×10^{-2}	2596	—31
	Ethanol	1×10^{-1}	2107	—44
	Ethanol	2×10^{-1}	1560	—69

TABLE VI. Attempts to Eliminate Inhibitory Effects of Chemicals on Antigenic Release of Histamine by Washing Leukocytes before Addition of Specific Antigen.

Chemical	Conc. (M)	Histamine release compared to controls (%)			
		Compound not removed	Cells washed		
			1 ×	2 ×	3 ×
Cyclic AMP	5×10^{-3}	—100	—54	—47	—50
Cyclic AMP	5×10^{-4}	— 58			—12
Theophylline	1×10^{-3}	— 77	—13	— 6	
Nicotinamide	2×10^{-2}	— 90	— 3		
Ethanol	2.5×10^{-1}	— 95	—13		

lease histamine, it was found that even after three washings leukocytes treated with cyclic AMP released about 50% less histamine than untreated leukocytes. In contrast, after one or two washings the inhibitory effect of the other compounds was nearly eliminated (Table VI).

Discussion. Antigen released histamine from leukocytes of hypersensitive subjects but not β-glucuronidase, whereas, during phagocytosis of zymosan particles, β-glucuronidase was released from leukocytes but not histamine. Similar observations have been made by others (9). In addition these compounds were found to inhibit phagocytic release of the lysosomal enzyme β-glucuronidase.

It is noteworthy that concentrations of the four compounds found to inhibit histamine release were of the same magnitude as required to suppress utilization of glucose by leukocytes, and the rank order of potencies was the same for both processes. The same sort of relationship between inhibitory capacities of these compounds has been observed with respect to their inhibition of the proliferative response of lymphocytes to stimulation by antigen or phytohemagglutinin (10).

The modes of inhibitory actions of the compounds remain to be determined, and in this connection some comments seem pertinent. The four compounds under consideration could each have a direct, primary inhibitory action on some crucial structure or function of leukocytes, and consequently reduce the need for utilization of glucose as an indirect or secondary effect. Among features of the cell upon which the compounds could act are: membranes, contractile proteins, flow and merger of lysosomes, and enzymatic reactions involved in transfer of energy and in synthesis and degradation of cell constituents. However, because some leukocyte functions are clearly dependent on metabolism of glucose (4), direct, primary disturbance of steps in glucose utilization could conceivably have a suppressive effect on cellular responses—as has already been demonstrated for histamine release (11) and suggested for uptake and retention of the dye neutral red by lysosomes (12).

The term "utilization" is purposely and appropriately applied to the interference with conversion of glucose to CO_2 by leukocytes that occurred in the presence of the four inhibitory compounds, pending precise localization of their actions. Proper utilization depends on many steps from transport of glucose across membranes through the numerous enzymatic reactions involved in derivation of energy by the cell from metabolism of glucose to CO_2 and the associated process of oxidative phosphorylation.

Summary. Antigenic release of histamine from leukocytes of hypersensitive persons is not accompanied by release of the lysosomal enzyme, β-glucuronidase. Likewise, histamine is not released during phagocytic release of β-glucuronidase. Both processes are inhibited by dibutyryl cyclic 3′, 5′-adenosine monophosphate, theophylline, nicotinamide, and ethanol. These four compounds were found to also suppress utilization of glucose by leukocytes from humans, the concentrations and

rank order of potency required being the same magnitude as inhibited release of histamine. Considerations pertinent to determination of mechanisms which could account for the effects of these compounds are discussed.

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1. Mota, I., Dias da Silva, W., and Fernandes, J. Ferreira, Brit. J. Pharmacol. **15**, 405 (1960).

2. Chakravarty, N., Acta Physiol. Scand. **48**, 146 (1960).

3. Lichtenstein, L. M., and Margolis, S., Science **161**, 902 (1968).

4. Karnovsky, M. L., Seminars Hematol. **5**, 156 (1968).

5. May, C. D., Levine, B. B., and Weissmann, G., Fed. Proc. **28**, 378 (1969).

6. Lichtenstein, L. M., and Osler, A. G., J. Exp. Med. **120**, 507 (1964).

7. Brittinger, G., Hirschhorn, R., Douglas, S. D., and Weissmann, G., J. Cell Biol. **37**, 394 (1968).

8. Leinweber, F.-J., and Walker, L. A., Anal. Biochem. **21**, 131 (1967).

9. Pruzansky, J. J., and Patterson, R., Proc. Soc. Exp. Biol. Med. **124**, 56 (1967).

10. May, C. D., J. Allergy **43**, 163 (1969). (Abstract).

11. Chakravarty, N., Nature **194**, 1182 (1962).

12. Hirschhorn, R., Brittinger, G., Hirschhorn, K., and Weissmann, G., J. Cell Biol. **37**, 412 (1968).

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