STUDIES ON LYSOSOMES

XII. Redistribution of Acid Hydrolases in Human Lymphocytes Stimulated by Phytohemagglutinin

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ABSTRACT

Subcellular fractions were isolated by differential centrifugation from pure suspensions of human blood lymphocytes incubated with and without phytohemagglutinin (PHA). Between 30 and 120 min after addition of PHA to intact cells, redistribution of acid hydrolases (beta glucuronidase, acid phosphatase), from a 20,000 $g \times 20$ min granular fraction into the corresponding supernatant, was observed. No increase in total acid hydrolase activity was found at these times. The mitochondrial marker enzyme, malate dehydrogenase, did not undergo redistribution. Granules derived from PHA-treated cells became more fragile upon subsequent incubation with membrane-disruptive agents in vitro (streptolysin S, filipin). These changes were associated with an increase in the over-all permeability of the stimulated cell to substances in the surrounding medium, such as neutral red. Augmentation of dye entry into lymphocytes required intact metabolism as judged by response to temperature and inhibitors (cyanide, antimycin A, 2,4-dinitrophenol). PHA, however, did not release enzyme activity from hydrolase-rich granules in vitro or render them more susceptible to subsequent challenge with membrane-disruptive agents. These studies suggest that PHA induces early changes in the surface of lymphocytes. The consequent redistribution of acid hydrolases may play a role in remodeling processes of the stimulated cells.

INTRODUCTION

The small lymphocyte of peripheral blood is a long-lived cell which remains morphologically and metabolically unaltered for extended periods (1). However, after the addition in vitro of agents such as phytohemagglutinin (PHA) (2–5), pokeweed mitogen (6–8), streptolysin S (9), or staphylococcal exotoxin (10), lymphocytes undergo transformation to large basophilic cells whose nuclei show active reticular chromatin, instead of the dense, inactive chromatin of the resting cell; they also display prominent nucleoli. These morphologic changes culminate in a peak of mitosis 72 hr after onset of stimulation, and recent studies have documented that they are accompanied by striking metabolic alterations in the stimulated cell. Thus, during the first hours after exposure to PHA, there are rapid increases in the synthesis of RNA (11, 14) and protein (12, 14), some of which is of a species not previously produced by the resting lymphocyte. Induced formation of enzymes active in RNA synthesis is also found (13). However, degradation of preexisting RNA (11), with increases in acetylation of nuclear histones (14) and turnover of phosphoproteins (15), can be detected even before

enhanced RNA and protein synthesis. Such changes closely resemble the consequences of similar, widespread gene activation found in regenerating liver (16). Much later, after these early phases of activation, and approximately 36 hr after lymphocytes have been exposed to PHA, new synthesis of DNA can be demonstrated (14, 17, 18).

The mechanism whereby PHA can initiate this transformation of lymphocytes remains unknown. However, studies on various compounds which can either activate (9, 10), or prevent the activation (19, 20) of lymphocytes have suggested that lymphocyte lysosomes may mediate some of the metabolic alterations observed in the transforming small lymphocyte (21). Histochemical studies at the light microscopic level have indeed indicated that lymphocyte lysosomes were "labilized" early in the course of PHA-induced transformation (22).

The preceding paper (23) has demonstrated the isolation, from unstimulated lymphocytes, of cytoplasmic granules which appear to possess many of the biochemical and morphologic properties of lysosomes. With similar techniques, the studies reported below demonstrate that shortly after PHA, there is a redistribution of acid hydrolases from granular fractions into fractions that could not be sedimented. Granules from stimulated cells were rendered more fragile to subsequent incubation with membrane-disruptive agents in vitro; such changes were associated with increases in the over-all permeability of stimulated lymphocytes to substances in the surrounding medium. PHA, however, had no direct effect upon isolated hydrolase-rich granules. These studies were compatible with the hypothesis that PHA induced early changes in the surface of the lymphocyte which secondarily led to alterations in the lysosomes of stimulated cells.

MATERIALS AND METHODS

Separative Procedures, Cell Counts, and Viability

The experiments were conducted with purified suspensions (>99%) of human peripheral blood lymphocytes obtained as previously described (23).

Culture Procedure

The cell suspensions were transferred to 50-ml screw-top, shallow conical glass centrifuge tubes (Bellco Glass Inc., Vineland, N. J.), and adjusted to a cell concentration of 1.5–2.5 × 10^6 per milliliter in a final volume of 40.0 ml with "complete" MEM Spinner medium. After preincubation for 180 min at 37°C, 0.8 ml of NaCl (0.9%) solution, or 0.8 ml of a phytohemagglutinin solution (phytohemagglutinin-P, PHA-P, from Difco Laboratories, Detroit, Mich., dissolved in 10.0 ml of sterile NaCl solution) was added. The cultures were then incubated for a further 30 or 120 min.

Homogenization and Fractionation

After having been harvested, cell suspensions were cooled, centrifuged, subjected to hypotonic lysis of red cells, and resuspended in 0.34 M sucrose containing 0.01 M EDTA, adjusted to pH 7.0 with 1 N NaOH, with or without 50 U of heparin per milliliter. Homogenization and fractionation was carried out as previously described (23). All fractions were stored at −18°C. Before biochemical analyses, the fractions and whole homogenates were treated with Triton X-100 (Rohm & Haas, Philadelphia, Pa.) at a final concentration of 0.1%. After standing for 15 min at 4°C, the preparations were centrifuged at 20,000 *g* for 20 min in a Sorvall Superspeed model SS-3 centrifuge. The supernatants were removed by pipette and used for biochemical analyses. Data are expressed as percentage of total recovered activity per 10^7 cells.

Biochemical Analyses

ENZYME ASSAYS: Beta: glucuronidase, acid phosphatase, acid ribonuclease, and malate dehydrogenase activities were determined as described previously (23).

Protein was measured according to Lowry et al. (24). A fine precipitate formed after addition of Folin-Ciocalteau reagent to the assay mixture, due to the presence of Triton X-100 in the homogenate. This was eliminated by centrifugation at 20,000 *g* for 10 min; the net absorbancy at 660 mμ was not affected by this procedure.

Release of Sedimentable Enzyme Activity

As in previous studies (23) agents to be tested were added in 0.04–0.05 ml aliquots to 0.76–0.95 ml aliquots of "postnuclear supernatants" containing both granular fractions and the cell supernatant. After incubation for 60 min at 37°C, the mixture was centrifuged at 20,000 *g* for 20 min, the supernatant was removed by pipette, and the pellets were adjusted to original volume with heparinized EDTA-sucrose solution containing 0.1% Triton X-100. Both supernatants and pellet suspensions treated with Triton X-100 were assayed for enzyme activity. In some experiments, the amount of enzyme activity which could not sediment in the postnuclear supernatant prior to any treatment was determined. Therefore aliquots were appropriately diluted with heparinized sucrose-

EDTA solution and immediately centrifuged at 20,000 *g* for 20 min. Release of enzyme activity by test compounds was then calculated by subtracting enzyme activities present in supernatants of untreated controls from enzyme activities present in supernatants of test samples. Results were expressed as (enzyme activity released into the 20,000 *g* supertant after incubation with Triton X-100) (× 100). The following compounds were used: streptolysin S (obtained from Dr. A. W. Bernheimer, New York University School of Medicine, New York) dissolved in phosphate buffer, 0.1 M, pH 7.4; filipin (The Upjohn Co., Kalamazoo, Mich.) dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, Mo.); and purified preparations of phytohemagglutinin (Burroughs Wellcome and Co., London, England; batch X-5 and preparation CT 1470, batch E-118) dissolved in 0.9% NaCl solution.

Uptake of Neutral Red

Neutral Red (National Aniline Corp.) was dissolved in isotonic sodium chloride solution to final concentrations ranging from 0.50 to 1.0 mg/ml. This solution (0.5 ml) was added to 5 ml of complete media containing from 10 to 12 × 10^6 lymphocytes. The cell suspension was incubated in a 37°C water bath, and harvested at various time intervals by placing the tubes in ice water after addition of 4 ml of ice-cold saline and then centrifuging at 1500 rpm (=503 *g* at tip of tube) for 10 min in an International model PR-2 refrigerated centrifuge at 4°C.

The cell pellet was then washed four times with aliquots of cold saline (4 ml), centrifuged at the same speed, resuspended in saline (2 ml), and frozen and thawed five times. The debris was sedimented at 2,000 rpm (=894 *g* at tip of tube) and the absorbancy of the neutral red in the clear supernatant was determined at 520 mμ in a Beckman DB spectrophotometer. The pH previously had been adjusted to less than 5.0 by the addition of 0.2 ml of 1.2 N HCl. The absorbancy of neutral red was linear with concentration. All metabolic inhibitors were dissolved in MEM-S and the pH was adjusted to 7.4 before addition in 0.1 ml aliquots. PHA-P was added in 0.1 ml aliquots to 5 ml of cell suspension 1 hr before addition of neutral red, unless otherwise indicated.

RESULTS

Separative Procedures, Cellular Viability, Enzyme Assays

As described previously (23), the separative method used resulted in cell preparations containing 99.8% mononuclear cells which represented almost pure suspensions of small lymphocytes. Although the majority of platelets could be removed from the plasma by treatment with ADP, a small and variable platelet contamination was still present in the final cultures. The viability of the cells, measured after 15 hr of standing at room temperature, averaged 95.4 ± 0.3% dye-excluding cells.

No acid hydrolase or malate dehydrogenase activities could be found in phytohemagglutinin preparations at concentrations of PHA present in the cultures (0.1 ml PHA-P solution per 5.0 ml culture medium). Only after a 10-fold increase of PHA-P concentration above the level used in cultures, could acid ribonuclease and malate dehydrogenase activities be detected; no beta glucuronidase or acid phosphatase activity was detected at these concentrations. Nor did streptolysin S and filipin, in appropriate concentrations, affect the enzyme assays.

After addition of PHA to the cultures, many agglutinated lymphocytes became adherent to the wall of the culture tube. During hypotonic lysis of erythrocytes with saline, a variable portion of these aggregates became resuspended. Despite vigorous addition of sucrose-EDTA with a Pasteur pipette to the final pellet during resuspension and subsequent homogenization, more cell aggregates were lost in PHA-treated cultures than in untreated cultures.

Effect of Incubation with Phytohemagglutinin for 30 Min

The levels of acid hydrolase and malate dehydrogenase activities have been described previously (23). In the present paper, the data have been expressed as enzyme or protein per 10^7 cells, to permit evaluation of changes in both enzymes and protein content. After incubation of lymphocyte cultures with PHA-P for 30 min, total activities of beta glucuronidase and acid phosphatase remained unchanged (Table I).

Enzyme distribution between granular and supernatant fractions is summarized in Table II. No differences could be detected in protein concentration or in malate dehydrogenase activity of fractions derived from PHA-treated and control-lymphocyte cultures. No significant differences were found between mean acid phosphatase activities of PHA-treated and control fractions. In PHA-treated homogenates, 19.1 ± 1.7% of recovered beta glucuronidase activity was found in

TABLE I

Total Recovered Enzyme Activity and Protein Content in Homogenates Derived from Lymphocyte Cultures Incubated with and without PHA-P for 30 Min at 37°C

Enzyme	No. of experiments		Activity Units ± SEM*	SED‡	Recovery ± SEM
					%
Beta glucuronidase	12	Control	18.86 ± 1.731	± 0.4472	89.9 ± 1.6
		PHA-P	18.77 ± 1.918		93.7 ± 2.4
Acid phosphatase	4	Control	149.8 ± 11.97	± 1.059	88.4 ± 6.0
		PHA-P	143.1 ± 12.61		92.7 ± 6.7
Malate dehydrogenase	4	Control	399.3 ± 51.05	±39.22	94.5 ± 6.2
		PHA-P	325.4 ± 67.78		99.1 ± 9.0
Protein	12	Control	523.0 ± 25.75	±22.69	117.5 ± 3.4
		PHA-P	490.6 ± 31.66		121.6 ± 5.4

* Activity Units (± Standard Error of Mean): beta glucuronidase, millimicromoles phenolphthalein released per 10^7 cells per hour; acid phosphatase, millimicromoles phosphorus released per 10^7 cells per hour; malate dehydrogenase, millimicromoles $NADH_2$ oxidised per 10^7 cells per minute; protein, micro g per 10^7 cells.

‡ Standard Error of Difference; control vs. PHA.

TABLE II

Distribution of Enzymes Among Subcellular (Granular and Supernatant) Fractions Derived from Lymphocyte Cultures Incubated with and without PHA-P for 30 Min at 37°C

Values are given as per cent of the total recovered activity ±SEM.

Enzyme	Treatment	No. of experiments	Granules	Supernatant	*t*-Test*
Beta glucuronidase	Control	14	54.1 ± 4.2	14.8 ± 1.2	$P < 0.05$
	PHA-P	11	47.7 ± 5.0	19.1 ± 1.7	
Acid phosphatase	Control	4	47.7 ± 3.5	25.5 ± 1.7	not significant
	PHA-P	4	39.5 ± 2.1	26.1 ± 3.0	
Malate dehydrogenase	Control	4	13.4 ± 0.7	67.7 ± 1.3	not significant
	PHA-P	4	17.4 ± 1.0	62.1 ± 1.3	
Protein	Control	14	14.2 ± 1.0	66.1 ± 2.3	not significant
	PHA-P	11	13.3 ± 1.4	63.4 ± 2.1	

* Control vs. PHA.

the 20,000 *g* supernatant whereas the supernatant of the corresponding control homogenates contained only 14.8 ± 1.2% of recovered activity; this difference was significant at the 0.05 level. A reciprocal, but insignificant, decrease of beta glucuronidase activity was discovered in the granular fraction of PHA-treated homogenates. In individual experiments, 7 of 11 cultures showed an increase of beta glucuronidase activity (per cent of recovered activity and relative specific activity) in the 20,000 *g* supernatant after incubation with PHA-P. Protein concentration and enzyme activities in the debris and nuclear fractions were not significantly different in PHA-stimulated and control lymphocytes. These data show that there is no consistent, significant redistribution of acid hydrolases 30 min after PHA stimulation. They are included in this report as a base line for changes occurring 120 min after stimulation, as a further indication of the reproducibility of the method, and in order to set a lower limit to the time-sequences involved.

TABLE III

Total Recovered Enzyme Activity and Protein Content in Homogenates Derived from Lymphocyte Cultures Incubated with and without PHA-P for 2 Hr at 37°C

Enzyme	No. of experiments		Activity units ± SEM *	SED‡	Recovery ± SEM
Beta glucuronidase	13	Control	17.26 ± 1.294	± 1.125	89.6 ± 2.5
		PHA-P	16.13 ± 1.310		98.4 ± 3.3
Acid phosphatase	8	Control	143.0 ± 12.31	± 8.180	99.7 ± 3.3
		PHA-P	138.8 ± 15.42		104.5 ± 5.7
Malate dehydrogenase	5	Control	373.5 ± 28.82	±10.31	107.8 ± 5.1
		PHA-P	302.1 ± 30.50		101.3 ± 7.3
Protein	13	Control	480.8 ± 24.58	±24.86	116.3 ± 5.3
		PHA-P	402.9 ± 34.06		114.3 ± 5.0

* Activity Units (± Standard Error of Mean): beta glucuronidase, millimicromoles phenolphthalein released per 10^7 cells per hour; acid phosphatase, millimicromoles phosphorus released per 10^7 cells per hour; malate dehydrogenase, millimicromoles $NADH_2$ oxidized per 10^7 cells per minute; protein, microg/10^7 cells.

‡ Standard Error of Difference, control vs. PHA.

TABLE IV

Distribution of Enzymes among Subcellular Fractions Derived from Lymphocyte Cultures Incubated with and without PHA-P for 120 Min at 37°C

Values are given as per cent of the total recovered activity ±SEM.

Enzyme	Treatment	No. of experiments	Fraction					
			Debris	Nuclei	Granules	P* ± SED	Supernatant	P* ± SED
Beta glucuronidase	Control	13	5.6±1.0	13.2±1.1	69.3±1.4	<0.001	12.0±0.87	<0.001
	PHA-P	13	3.6±0.5	18.7±2.8	57.8±2.3	±2.45	19.9±0.89	±0.774
Acid phosphatase	Control	8	4.9±1.6	15.7±2.0	61.6±2.7	<0.05	17.8±1.4	<0.001
	PHA-P	8	3.7±0.8	18.5±3.3	53.9±2.4	±3.21	23.9±1.4	±1.10
Malate dehydrogenase	Control	5	3.0±2.2	13.0±2.2	27.5±1.9	not significant	56.5±2.5	not significant
	PHA-P	5	3.5±0.9	16.3±4.0	31.3±2.9	±3.44	49.0±2.0	±2.41
Protein	Control	10	9.6±1.7	10.0±1.2	16.6±1.7	not significant	63.9±3.2	not significant
	PHA-P	10	7.2±1.4	11.5±1.5	16.2±0.9	±1.80	65.0±2.3	±2.28

* Paired *t*-test, ± standard error of differences between paired samples.

Effect of Incubation with Phytohemagglutinin for 120 Min

Compared to controls, no significant changes in total acid hydrolase activity per 10^7 cells were noted in PHA-treated cultures. Some decrease of malate dehydrogenase and total protein was evident (Table III) after 120 min.

In homogenates derived from lymphocyte cultures incubated for 120 min with PHA (Table IV), there was a marked increase of recovered beta glucuronidase activity in the 20,000 *g* supernatant ($P < 0.001$). Simultaneously, a decrease was observed of recovered beta glucuronidase activity in granular fractions of PHA-treated homogenates ($P < 0.001$). Nuclear and debris fractions remained unaffected. The activity of acid phosphatase showed the same distribution pattern. Differences

TABLE V

Influence of Purified Phytohemagglutinin on Sedimentable Enzyme Activity in Postnuclear Supernatant Derived from Untreated Lymphocyte Cultures

Samples were incubated for 1 hr at 37°C. Solvent, 0.9% NaCl. Calculation see text.

Agent	Dose	No. of experiments	Beta glucuronidase	
			Agent	Solvent
	μg			
Phytohemagglutinin-P (Difco Laboratories)	0.250	1	9.0	20.0
	0.625	1	13.8	20.0
	1.250	1	11.5	20.0
Phytohemagglutinin (Burroughs Wellcome and Co.) Batch X-5	1.0	1	18.3	19.0
	10.0	3	33.1	26.5
	100.0	3	32.9	26.5
	1000.0	2	37.1	30.3
Phytohemagglutinin (Burroughs Wellcome and Co.) Prep. CT 1470 batch E-118	0.1	1	20.0	19.1
	1.0	1	20.9	19.1
	10.0	1	10.0	19.1

in acid phosphatase activity between PHA-treated and control homogenates were significant at the 0.001 level for the 20,000 *g* supernatant and at the 0.05 level for the granular fraction. Mean malate dehydrogenase activities and protein concentrations were unchanged in all fractions of PHA-treated homogenates as compared to control preparations. In this series of experiments, the increase of beta glucuronidase and acid phosphatase activities (per cent of recovered activity and activity per 10^7 cells) in the 20,000 *g* supernatant could be detected in *all* individual experiments. In contrast, there was no redistribution of malate dehydrogenase, in any of the homogenates, from fractions that could be sedimented to those that could not.

Influence of Platelet Contamination

As described previously (23), contamination of lymphocyte cultures with platelets had only minor effects on the activity of beta glucuronidase and acid phosphatase in whole homogenates and subcellular fractions. Therefore, it was of interest to investigate whether the addition of PHA to lymphocyte cultures contaminated with platelets would have further effects on hydrolase activities. A platelet-rich suspension was prepared as described previously (23). By adding an appropriate aliquot of this preparation to a lymphocyte culture poor in platelets, an eight fold increase of platelet number was achieved. This culture and a platelet-poor culture were preincubated for 180 min at 37°C. Then, the usual amount of PHA-P was added to both cultures and these were again incubated for 120 min at 37°C .After having been harvested, the cultures were processed, homogenized and fractionated as usual. After fractionation, the total recovered beta glucuronidase activity of the platelet-rich, PHA-treated preparation was increased by a factor of 1.1, acid phosphatase activity by a factor of 1.7, and protein by a factor of 1.87 over corresponding activities in the platelet-poor preparation. In the platelet-rich homogenate, a slight increase of both beta glucuronidase and acid phosphatase activities in the 20,000 *g* supernatant was found whereas the other fractions were unaffected. Therefore even an exaggerated platelet contamination of PHA-treated lymphocyte cultures had only a minor effect on the activity of acid hydrolases in whole homogenates and the distribution of these enzymes among subcellular fractions.

Direct Effect of Phytohemagglutinin on Lymphocyte Granules

The redistribution of hydrolases described above could be explained if PHA directly released enzymes from hydrolase-rich organelles. Therefore PHA-P was added to lymphocyte lysosomes. These preparations induced immediate clumping of granule fractions in sucrose. Indeed, PHA-P appeared to retard the release of hydrolases into suspending media (Table V). Experiments in

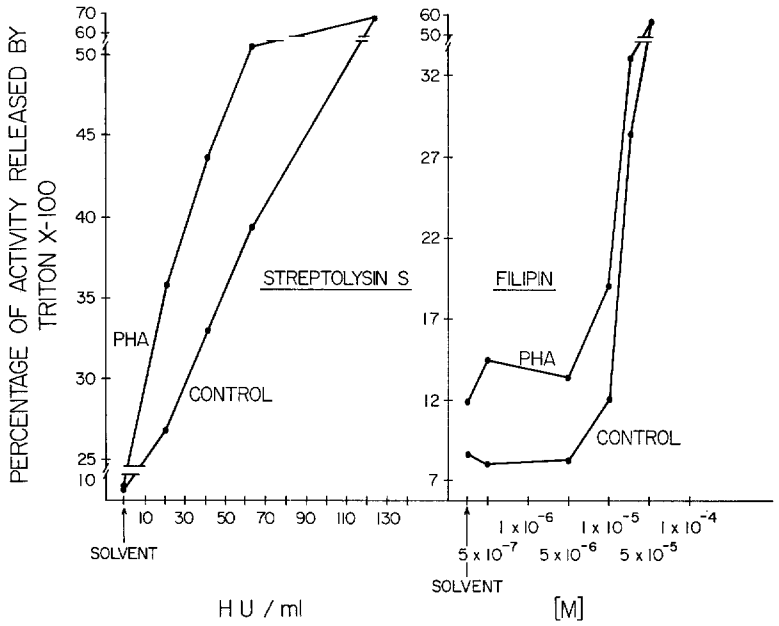

FIGURE 1 Release of beta glucuronidase from lymphocyte granules. Lymphocytes were incubated for 2 hr with and without phytohemagglutinin. Postnuclear supernatants were isolated (see text) from each of these, incubated for 60 min at 37°C with varying concentrations of streptolysin S and filipin, and beta glucuronidase activity in the 20,000 *g* supernatant was determined.

which PHA-P was added to large granule fractions of rabbit liver in 0.25 M sucrose (Weissmann, G. Unpublished data.) also showed that PHA-P prevented adequate mixing of the aggregates with the suspending media and thereby appeared to prevent the release of soluble enzyme. Therefore, the effects were studied of purified phytohemagglutinin preparations, which did not produce granule clumping, on enzyme release from granules present in a postnuclear supernatant from untreated lymphocytes. The results of these experiments are given in Table V. Phytohemagglutinin, Burroughs Wellcome and Co., preparation CT 1470, batch E-118, was a highly purified preparation, the mitogenic potency of which was approximately 180-fold higher than that of PHA-P (manufacturers specification) (Hirschhorn, R. Unpublished observations.). With various concentrations of this preparation, no release of *sedimentable* beta glucuronidase activity into the supernatant could be observed. Burroughs Wellcome and Co., batch X-5 phytohemagglutinin had an approximately 13-fold higher mitogenic potency than PHA-P (25) (Hirschhorn, R. Unpublished observations.). Only minimal release of beta glucuronidase activity was induced by this preparation. However, this release was not dose-dependent. These results make it unlikely that PHA exerts disrupting effects directly upon lymphocyte lysosomes.

Release of Hydrolases by Membrane-Active Agents

Streptolysin S and filipin, two membrane-disruptive agents, were added to postnuclear supernatants from untreated and PHA-P–stimulated lymphocytes, and the release of acid hydrolase activity was studied. Fig 1 summarizes the data of five separate experiments. Streptolysin S caused a dose-dependent release of beta glucuronidase activity from the control and PHA-P–treated organelles. Indeed, low concentrations of streptolysin S induced greater release of enzyme from PHA-P–stimulated granules than from controls. This difference was not detectable if higher, more disruptive concentrations of streptolysin S were employed. The response to filipin revealed the same pattern: augmented release of beta glucuronidase activity from PHA-P–treated granules. With streptolysin S, malate dehydrogenase activity in the 20,000 *g* supernatant rose to a variable ex-

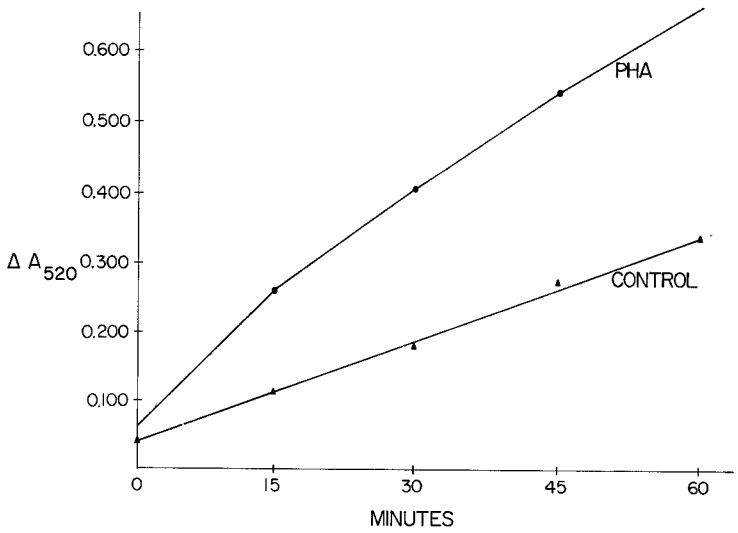

FIGURE 2 Uptake of neutral red by lymphocytes. Lymphocytes were incubated with and without phytohemagglutinin for 1 hr, and neutral red was added at a final concentration of 58 μg/ml. Cells were washed, and neutral red remaining in the supernatant of a cell lysate was determined. Results are expressed as (A) = Absorbance at 520 mμ at acid pH.

tent. No dose-dependence was observed for malate dehydrogenase release, nor was there an increased response in PHA-P–treated preparations. In both preparations, as expected, malate dehydrogenase release remained unaffected by addition of filipin (26), since this polyene antibiotic preferentially disrupts lysosomes but has *no* effect upon mitochondria. PHA pretreatment of isolated control granules did not result in a greater susceptibility of these organelles to streptolysin S and filipin.

Uptake of Neutral Red

From the previous experiments it appeared that redistribution of acid hydrolases after addition of PHA was not the result of a direct action of the agent upon acid hydrolase–containing organelles. Since endocytosis may lead to the formation of newly formed fragile lysosomes (see Discussion), the effect of PHA upon endocytosis by lymphocytes was next examined. The phenazine dye neutral red (molecular weight, 288.8) was used as a marker. This dye has been shown to be concentrated within lysosomes after transport across the cell membrane, but it is not clear whether the dye enters the cell by simple diffusion and is then concentrated within lysosomes (either by active transport or autophagy), or is taken up by micropinocytosis with subsequent fusion of the pinocytic vacuoles with lysosomes (27).

Lymphocytes were incubated in neutral red–containing solution, and examined microscopically within 1 min. One to two tiny red granules were observed in almost all cells; rare cells contained several granules. There was a faintly pink cast to the cytoplasm. Over the next 2 hr, both the number and size of the neutral red–containing granules increased to five to six per cell. However, by light microscopy it was not possible to determine whether any change occurred following PHA stimulation, since the cells could not be spread sufficiently Therefore the amount of neutral red accumulated by lymphocytes was measured spectrophotometrically. As seen in Fig. 2, the amount of neutral red accumulated by unstimulated lymphocytes showed a linear increase with time up to 1 hr. If the lymphocytes were kept at 4°C for this period or killed by incubation at 56°C for 1 hr dye accumulation was markedly reduced (Fig. 3). Uptake and retention of neutral red could also be inhibited by 5×10^{-4} M cyanide, 1.68×10^{-7} M antimycin A (Fig. 3) and 2×10^{-4} M 2,4 dinitrophenol, although it was unaffected by sodium fluoride or iodoacetate. Thus, the transport and/or retention of neutral red by lymphocytes appeared to be an energy-requiring process, and was dependent upon viability, temperature, respiration, and oxidative phosphorylation, although not upon glycolysis. Consequently, the effect of PHA stimulation upon

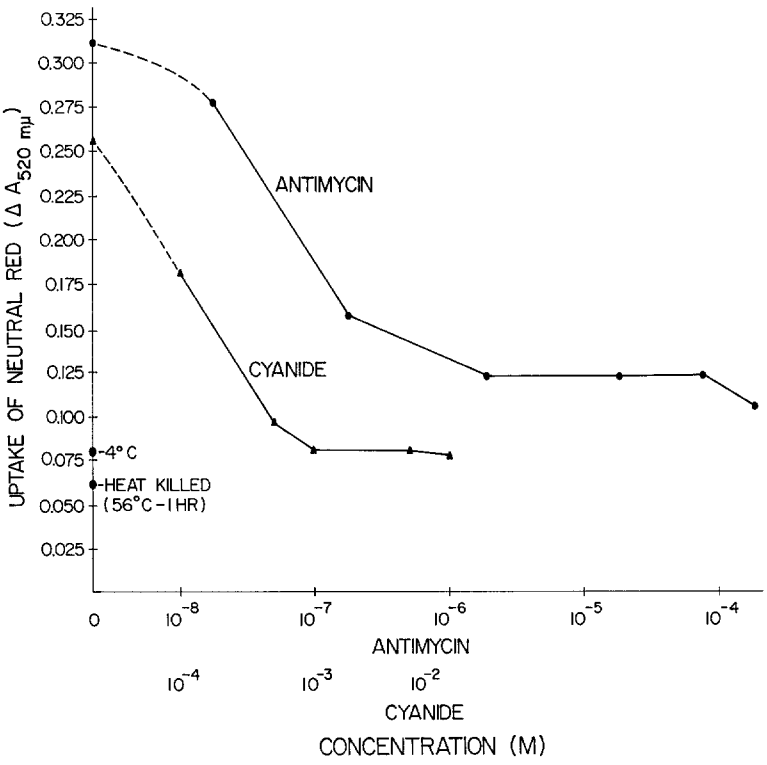

FIGURE 3 Inhibition of neutral red uptake in lymphocytes. Lymphocytes were incubated for 45 min with neutral red (58 μg/ml) (see text). Cyanide, antimycin A, saline, or ethanol was added at time 0. Cells were also kept at 4°C throughout the experiments or pretreated at 56°C for 1 hr. Cells were washed, and neutral red remaining in the supernatant of a cell lysate was determined. Results are expressed as (A) = Absorbance at 520 mμ at acid pH.

neutral red accumulation by lymphocytes was studied. PHA was added to prewarmed lymphocytes 1 hr before addition of neutral red; at this time there was no difference in total acid hydrolase content between control and stimulated lymphocytes. There was a twofold increase in the amount of neutral red accumulated at 60 min following addition of the dye, or 2 hr after PHA (Fig. 2). This increased accumulation was also evident within 20 min after PHA stimulation and, indeed, the *rate* of dye accumulation appeared to be unchanged during the first 2 hr after PHA.

DISCUSSION

The data presented above demonstrate that during early phases of lymphocyte stimulation by PHA the *total* enzyme activities per cell of two acid hydrolases (beta glucuronidase, acid phosphatase) do not differ significantly from controls. Nevertheless, this phase is characterized by an increase of acid hydrolase activity in the 20,000 g supernatant, accompanied by a corresponding decrease of acid hydrolase activity in the particulate (granular) fraction. Such redistribution of enzyme activity appears to be specific for lysosomal hydrolases since the mitochondrial marker enzyme, malate dehydrogenase, does not undergo redistribution. These data suggest that there was no net increase in acid hydrolase content of lymphocytes 30 and 120 min after PHA, although there was loss of protein and malate dehydrogenase activity at the later interval. It cannot be excluded that newly synthesized acid hydrolases were retained by these cells, some of which had been rendered as permeable to protein as to neutral red. However, were newly synthesized enzymes to appear free in the cytoplasm, they would be expected to be lost *pari passu* with proteins, thereby decreasing the percentages recovered in supernatants. On the other hand, were newly synthesized granules formed which sedimented at 20,000 g, the percentage of activity in the granular fraction would

be increased. Changes in these directions were not found. However, it remains possible that smaller, hydrolase-rich granules were formed which were incapable either of being extruded from the cell or sedimented at 20,000 *g*.

Data from this and earlier studies (23) indicate that the fractionation procedure was sufficiently reproducible to permit judgment as to whether redistribution of enzyme activity among subcellular fractions did in fact take place. Indirect proof of this can be adduced from the experiments in which cells were incubated for only 30 min with PHA, since these samples were directly comparable to control samples after 2 hr incubation.

Increased activity of unsedimentable acid hydrolases could result from actual release of enzymes into the cell sap of intact lymphocytes, or to an augmented liberation of enzymes from hydrolase-rich granules during homogenization and fractionation procedures. On the basis of the available data, neither alternative can be excluded. Release of enzymes occurring only during the preparative procedure would be due to increased mechanical fragility of hydrolase-rich organelles stimulated with PHA. Increased mechanical fragility has indeed been observed in autophagic rat liver lysosomes induced by injection of glucagon (28). Increases in osmotic and mechanical fragility have also been found in the phagolysosomes formed after administration of Triton WR-1339 (29). Weissmann and Uhr (30) found that phagolysosomes formed after administration of thorotrast were also more easily disrupted in vitro, and suggested that leakage of beta glucuronidase into cell sap and circulation explained elevations of enzyme activity in serum. Thus, a variety of autophagic or heterophagic stimuli lead to the formation of more "fragile" secondary lysosomes (31). Compared to hydrolase-rich organelles originating from untreated cells in vitro, granules from PHA-stimulated lymphocytes exhibit striking changes in surface properties. These granules showed an increased susceptibility to the disruptive action of such membrane-active agents as streptolysin S and filipin. Whether release of acid hydrolase activity from granules (increased mechanical fragility?) and the increased susceptibility of the remaining granules to membrane-disrupting agents reflect a common, primary effect of PHA or even occur among the same population of granules, requires further study. Another possibility, discussed above, is that PHA induced the formation of a new granule population, the sedimentation properties of which resembled those of "microsomes," (which cannot be sedimented at 20,000 *g* for 20 min). Thus, enzymes found in supernatant fractions might be associated with smaller, less dense particles which would contain newly synthesized enzymes (primary lysosomes?). Judging by 95% confidence limits of the differences, we could not, for example, exclude the possibility that PHA-treated cultures contained 7.65% more total beta glucuronidase activity than control cultures. Were *all* of this activity to have been recovered in the supernatant, it would barely suffice to explain both the increments in enzyme activity recovered in the supernatants and the decrements in granular fractions. This would require *no* loss of recoverable activity at all, and should be accompanied by consistent decreases of percentages in debris and nuclear fractions; these have not been found. Furthermore, we have shown the granules of PHA-treated cells to be more fragile to membrane-disruption. Were all the changes in distribution of hydrolases due to new synthesis, an *independent* mechanism would have to be postulated for the subsequent lability of granules from PHA-treated lymphocytes, since newly formed organelles would not be present in the large granule fractions but would have to remain in the supernatants. Although new synthesis of enzymes cannot be excluded as responsible for the observed changes, it would appear more likely that both lability of isolated granules and enzyme redistribution reflect a common, primary action.

The exact mechanism by which lysosomal hydrolases are redistributed and granules are rendered fragile during the early phase of stimulation with PHA is not readily apparent. However, both direct and indirect actions of PHA upon hydrolase-rich granules are possible. It was shown that incubation of hydrolase-rich organelles with purified PHA did not result in a dose-dependent release of beta glucuronidase activity. Therefore, PHA did not appear to exert a direct action on hydrolase-rich granules. Furthermore, when isolated granules were preincubated with purified PHA, and subsequently challenged with membrane-disrupting agents, the PHA-treated granules were not more susceptible to disruption than controls. Therefore, it is unlikely that PHA exerts its effect upon hydrolase-rich granules of stimulated cells directly, and may require for its action mechanisms operative only in intact cells. Cohn

and Wiener (32, 33), studying phagocytizing rabbit macrophages, showed that homogenates of these cells contained a higher proportion of acid hydrolases in soluble form than homogenates of resting cells. Therefore, it is entirely possible that transfer of lysosomal enzymes from preexisting granules to larger vacuoles during formation of secondary lysosomes would be accompanied by an increased fragility of the latter organelles, and thus lead to their rupture during homogenization of the cell.

Our data show that during the early phase of lymphocyte stimulation by PHA the over-all uptake of neutral red was enhanced. Judged by the effects of several metabolic inhibitors, dye uptake was demonstrated to be due to an active, energy-dependent process, and reflected enhanced transfer and/or concentration of neutral red into PHA-stimulated lymphocytes. Since increased endocytosis gives rise to enhanced formation of heterolysosomes (32, 33), these newly formed organelles could be more susceptible to mechanical disruption than preexistent hydrolase-rich granules. Therefore, redistribution of acid hydrolases might be due to the merger of primary lysosomes with endocytic vacuoles and formation of secondary hydrolase-rich granules which follow the increased endocytic processes of the stimulated cell. Alternatively, PHA may have induced the formation of structures more readily capable of concentrating both dye and hydrolases, structures which would also be more susceptible to the trauma of fractionation. At the present time, no evidence is available concerning the mechanism underlying this enhanced accumulation of neutral red, and these mechanisms are now under study.

We have previously reported that lymphocytes transformed by PHA become rich in newly formed lysosomes (34). This increase in acid phosphatase–positive, membrane-bounded structures was maximum at 48–72 hr, and thus reached a peak immediately before mitosis. Concurrent with an increase in demonstrable organelles, these enlarged lymphocytes had considerable increases in the *total* activity of acid phenolphthalein phosphatase, acid beta glycerophosphatase, and aryl sulfatase.[1] With cruder fractionation techniques than those employed in the present studies, the acid hydrolases were found to be membrane-bounded. This increase of lysosomes *late* in PHA-induced transformation (48–72 hr) may be a consequence of, or a response to, the *early* labilization of lysosomal hydrolases (2 hr after PHA). This connection, however, is not definable at present.

The data presented in this paper show that, during the early phase of stimulation of lymphocytes by PHA, labilization of hydrolase-rich organelles takes place, and the data directly support the hypothesis that lysosomal enzymes might play a role in the derepression of resting lymphocytes and their subsequent remodeling processes (21). It is possible, for example, that the early degradation of preexistent RNA in stimulated lymphocytes which was observed by Cooper and Rubin (11) is due to the action of nucleases. Furthermore, the hydrolysis of repressor proteins of the lymphocyte nucleus may well be the means whereby this widespread gene activation is brought about. Indeed, we have recently observed that nuclei from PHA-treated cells have increased template activity for exogenous RNA polymerase (35). In sum, the changes in cell surface induced by PHA are accompanied by both cytoplasmic and nuclear changes. Whether lysosomal hydrolases mediate these changes as a primary consequence of PHA action or are secondary markers of other events is as yet unclear.

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[1] Data for total acid hydrolase content in reference 34 is directly comparable with results of the present studies, e.g. acid beta glycerophosphatase was 159.3 mμmoles $P_i/10^7$ cells per hr at 0 hr vs. 143.0 mμmoles $P_i/10^7$ cells per hr after 2 hr (Table III). Fractionation data are not directly comparable, since the preparative techniques differed considerably.

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