Appearance of Hydrolase Rich Granules in Human Lymphocytes Induced by Phytohemagglutinin and Antigens

By Rochelle Hirschhorn, Kurt Hirschhorn and Gerald Weissmann

When human lymphocytes obtained from either peripheral blood or thoracic duct lymph are cultured in vitro, they remain in a resting state for several days. From the work of numerous laboratories (see ref. 1), it would appear that there are two classes of agents which stimulate lymphocytes to undergo mitosis between 48 and 72 hours after addition. These are (1) “non-specific” agents such as phytohemagglutinin (PHA), streptolysin S (SLS), antileukocyte antibodies, staphylococcal exotoxins, and (2) “specific” agents such as antigens to which the donors have previously been sensitized, allogeneic cells, or allogeneic cell extracts. Before mitosis, the stimulated lymphocytes undergo several metabolic and morphologic changes. After exposure to PHA, lymphocytes clump and promptly begin the synthesis of RNA2 and protein, a small percentage of which is gamma globulin. By 24 hours the nuclear chromatin has begun to change from repressed, or heterochromatin, to active, or euchromatin. After approximately 36 hours in culture, the lymphocytes begin to synthesize DNA, culminating in a mitotic peak at approximately 72 hours. Before cell division, 75 to 95 per cent of the cells have already enlarged, becoming basophilic, pyroninophilic, and PAS positive. They possess a perinuclear clear zone, and acidophilic granules have become visible in many of these cells.

Several investigators3-4 have documented the appearance and/or redistribution in various other cell types of acid phosphatase-rich granules shortly before mitosis, raising the possibility that lysosomes play a role in the mitotic process. Since PHA-stimulated lymphocytes develop a perinuclear clear zone, possibly representing an enlarged Golgi apparatus, and acidophilic granules, it appeared quite likely that new, lysosome-like structures had arisen in the premitotic lymphocyte. Novikoff5 had previously suggested that lysosomes take their origin from the small vesicular bodies of the Golgi apparatus, and Coulson6 has recently reported an increased Golgi formation in stimulated lymphocytes.

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HYDROLYASE RICH GRANULES IN HUMAN LYMPHOCYTES

In a preliminary report, we have shown an increase of acid phosphatase activity (biochemically and histochemically) in cultures of PHA-stimulated lymphocytes which were "contaminated" by the presence of polymorphonuclear leukocytes. The experiments reported below, utilizing cultures of pure lymphocytes, have demonstrated that this is a net increase, accompanied by a net increase in aryl sulfatase activity, and have provided evidence that this enzymatic activity is associated with membrane-bounded granules. In contrast, the activity of another lysosomal enzyme, \( \beta \)-glucuronidase, was shown not to increase. The effects upon lymphocyte acid phosphatase activity of specific antigens and SLS were also examined, as was the effect upon lymphocyte cultures of a lysosomal "stabilizer," chloroquine. It was found that both specific antigen and SLS induced the appearance of acid phosphatase-rich granules, whereas chloroquine inhibited the formation of granules induced by PHA.

**Materials and Methods**

**Separative Procedures**

Suspensions of 92-99 per cent pure lymphocytes were used in all experiments assayed biochemically. These were obtained using a column consisting of the thin portions of three nylon stockings (regular weave, 400, 15 Verlaine, light-colored, washed with 7X Linbro Chemical Co.) as for tissue culture) packed into a 50 ml. syringe with a small amount of washed nylon wool from a Leukopak (Fenwal Co.) placed at the bottom of the syringe. The assembled column was autoclaved and dried in a 37 C. incubator. Human, heparinized blood was sedimented at 37 C., the supernatant plasma removed, and 50 ml. of the plasma suspension was incubated on the prewarmed column at 37 C for 10 minutes. It was then collected at the rate of 30 drops per minute through a stopcock into a bottle covered with a rubber diaphragm. The column was washed with one and a half volumes of MEM Spinner medium (MEMS, Grand Island Biological Co.), using the same collection rate. The cells were washed three times in MEMS by centrifugation at 600 r.p.m. for 10 minutes in an International Universal Model UV Centrifuge to eliminate platelets. The purity of the resulting cell suspensions was determined by differential counts on sediments which had been resuspended in a drop of serum, smeared, and stained with tetrachrome stain.

For some of the histochemical studies, another, previously described method for separating lymphocytes was used, based upon nonadherence of lymphocytes to glass. Preparations varying in purity from 75-95 per cent were used. Non-mononuclear cells were not included in enumeration of stained and unstained cells.

**Culture Procedures**

Cells were cultured in 15-25 ml. aliquots of MEM Spinner media, supplemented with 20 per cent fetal calf serum, 1 per cent 200 mMol L-glutamine, 100 U. penicillin G, and 100 \( \mu \)g. streptomycin per ml., in Bellco screw-top, shallow, conical, centrifuge tubes placed in a 37 C. water bath. The cultures contained from 7.5 to 10 \( \times \) 10\(^6\) mononuclear cells per ml. Two lots of MEMS were used throughout, after four different commercial lots had been screened for their capacity to maintain good cellular viability. Several different lots of fetal calf serum were used. Replicate tubes were removed at appropriate time intervals. Phytohemagglutinin M (Difco) was added, 0.3 ml. per 15 ml. of culture. Streptolysin S (obtained from Dr. A. S. Bernheimer) was added as 100 H.U. per 5 ml. of culture. Purified protein derivative of tuberculin (PPD, Farke Davis Co.) was added as 2.5 \( \mu \)g. per 5 ml. of culture.
Cellular Morphology and Viability

Large cell formation and mitotic rate were determined on replicate tubes by the method of Hirschhorn after cells were exposed for 2 hours to vinblastin (Velban, Eli Lilly), 0.05 μg per 5 ml. of culture. Viability was determined using erythrocin B at a final concentration of 0.067 per cent. White blood cell counts were determined using a 1:1 dilution of cells with appropriately diluted counting fluid.

Biochemical Analyses

Cells for biochemical assay were washed 3 times in ice cold 0.25 M sucrose and then rapidly frozen and thawed 6 times. The supernatant culture media and sucrose washes were assayed where indicated. Medium without cells was incubated at 37 C. for the appropriate time interval to serve as a blank, since there was enzyme activity in the fetal calf serum which was lost during incubation at 37 C.

Beta-glucuronidase activity was assayed by the method of Talalay et. al., using 0.2 ml. of homogenate, 0.6 ml. acetate buffer (pH 4.6, 0.1 M), and 0.04 ml. of substrate (Sigma, 0.01 M solution phenolphthalein glucuronidate). The reaction was stopped after 4 hours of incubation at 37 C. with 2 ml. of glycine buffer, pH 10.4, 0.2M in 0.2M NaCl. Activity in the culture media was assayed for 5-24 hours.

Acid phosphatase activity was determined by a modification of the method of Valentine and Beck, using 0.2 ml. homogenate, 1.8 ml. 0.052 M β-glycerophosphate (Sigma) in acetate buffer, pH 5.0, 0.05M. The reaction was stopped after 4 hours incubation at 37 C. with 0.4 ml. of ice cold 35 per cent trichloracetic acid and then was filtered. Although the optimal substrate concentration was determined to be 0.05 M β-glycerophosphate, it was found that, using Fisk and Subbarow determination for inorganic phosphorous (P_i), the large aliquots of reaction mixture resulted in a final substrate concentration which inhibited early formation of the chromogen, while yielding an increasing amount of chromogen over the next 8 hours. A final concentration of 0.014 M β-glycerophosphate in the Fisk and Subbarow reaction mixture reduced the rate of color formation during the first 10 minutes. A final concentration of 0.035 M β-glycerophosphate reduced the reading to 30 per cent of the standard alone, even at 30 minutes. Therefore, P_i was determined by the method of Chen and Toribara, modified so that 0.3 ml. of deproteinised enzyme-substrate mixture added to 0.7 ml. of assay solution yielded the same final concentrations described in the original method. This concentration of substrate in the reaction mixture had no effect on color formation. Acid phosphatase activity was also determined by the method of Huggins and Talalay, using phenolphthalein diphosphate as substrate, against standards of acid phosphatase (Worthington Biochemical Co.). Two-tenths of a ml. of homogenate was added to 1.0 ml. of buffered substrate, incubated for 4 hours at 37 C. Media was assayed for 4 to 24 hours.

Aryl sulfatase was assayed by a modification of the method of A. B. Roy, using 0.2 ml. of homogenate, 0.4 ml. of 0.01 M nitrophenyl sulfate (Sigma), and 0.2 ml. acetate buffer, pH 5.0, 0.5 M. The reaction was stopped with 1 ml. 2 per cent phosphotungstic acid in 0.1 N HCl and the protein-free filtrate was diluted 1:1 with developing solution. Because of the low initial enzyme activity, it was necessary to assay for 24 hours at 37 C. The enzyme activities after 24 hours of assay were proportional to increasing lymphocyte concentrations over the range used.

Protein content was determined by the method of Lowry et. al., using standards of Pro-Sol (Dade Chemical) solution, which had been stored at 0 C.

Cell Fractionation

Cells from 6-8 tubes were harvested at 48 hours, centrifuged at 1500 r.p.m. for 15 minutes in an International Universal Model UV Centrifuge, washed twice in ice cold 0.25 M sucrose and resuspended in 3 ml. of 0.34 M sucrose. They were homogenized by 50-100 rapid expulsions through a Pasteur pipette, followed by centrifugation at 112 g and further homogenization of the pellet with a teflon and glass homogenizer in an addi-
tional 1 ml. of 0.34 M sucrose. Cell lysis was followed with phase microscopy, as well as by initial and final cell counts. Estimates of per cent cell lysis were, at best, approximate because of the inherent difficulties in counting cells which have been aggregated by phytohemagglutinin. In order to obtain a granule fraction uncontaminated by nuclei and debris, 3 ml. of homogenate were centrifuged at 1000 g for 10 minutes in 5 ml. lusteroid tubes in a Servall Automatic Superspeed C Centrifuge. The pellets, which contained nuclei, debris, and some large granules, were washed with 1 ml. 0.34 M sucrose and the supernates were centrifuged at 15,000 g for 20 minutes. Both pellets were resuspended to 2 ml. in 0.34 M sucrose. Aliquots of each fraction (including the supernate of the 15,000 g spin) were frozen and thawed 5 times, and enzyme activity and protein content were determined.

Release of Sedimentable Enzyme Activity

One ml aliquots of each of the fractions obtained were incubated with and without 1×10^{-8} M lyssolecithin (Mann Research Chemicals) for 30-45 minutes at 24°C and then centrifuged at 15,000 g for 20 minutes. The resulting pellets were resuspended to their original volume. Aliquots of the pellets, supernates, and total fractions were frozen and thawed 5 times and enzyme activities were determined. Results are expressed as the activity released into the 15,000 g supernate after incubation either with or without lyssolecithin/enzyme activity of the total fraction after incubation × 100.

Histochemistry

Acid phosphatase activity was localized histochemically, as previously described, using cultures of lymphocytes separated both by the nylon and the glass-bottle technic. Cultures were harvested at appropriate intervals, centrifuged, and resuspended in one drop of serum. They were spread on glass slides, fixed at 4°C in 10 per cent acetate-buffered, neutral formalin for 1 hour and incubated for 5 hours in standard Gomori substrate (β-glycerophosphate, containing less than 0.1 per cent of the α-isomer). Grain counts for 500 cells were performed on coded slides in most experiments.

Chloroquine

In experiments using chloroquine (Resochin, Bayer Leverkusen), purified lymphocyte cultures were obtained and chloroquine diphosphate added to a final concentration of 5×10^{-4} mg/ml. at zero hour. The chloroquine was previously diluted in MEMS, the buffering effect of which maintained the original pH of the culture.

RESULTS

Separative Procedures and Cellular Viability

The separative method used resulted in suspensions containing 92-99 per cent lymphocytes, as determined from tetrachrome-stained smears. Under the culture conditions used, as seen in Table 1, there was no significant change in cell counts of control cultures during the 72 hours of culture (as determined by replicate cell counts), nor was there an appreciable rise in non-viable cells as determined by staining with erythrocin B. Judged by the percentage of enlarged cells, and the mitotic index, no significant mitogenesis was observed in control cultures. Cell counts from cultures to which PHA was added showed a marked drop to 56 per cent of the zero hour count by 4 hours. However, if these cultures were centrifuged at 1000 r.p.m. for 10 min., treated with 0.7 per cent citrate and pipetted repeatedly to disperse clumps, re-centrifuged at 1000 r.p.m. for 10 min., and finally resuspended to the original
Table 1.—Cell Counts, Viability, Large Cell Formation and Mitotic Index in Human Peripheral Blood Lymphocytes Cultured with and without Phytohemagglutinin (PHA)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PHA</th>
<th>0 hrs.</th>
<th>4 hrs.</th>
<th>24 hrs.</th>
<th>48 hrs.</th>
<th>72 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Count *</td>
<td>100%</td>
<td>104%</td>
<td>100%</td>
<td>103%</td>
<td>99%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(% of 0 Hrs.)</td>
<td>PHA</td>
<td>100%</td>
<td>54%</td>
<td>23%</td>
<td>28%</td>
<td>98%</td>
<td></td>
</tr>
<tr>
<td>Viability †</td>
<td>Control</td>
<td>0.8%</td>
<td>0.4%</td>
<td>4.0%</td>
<td>2.0%</td>
<td>1.5%</td>
<td></td>
</tr>
<tr>
<td>(% non-viable cells)</td>
<td>PHA</td>
<td>0.8%</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>“Large cells” ‡</td>
<td>Control</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>4.8%</td>
<td>2.1%</td>
<td></td>
</tr>
<tr>
<td>(% of total)</td>
<td>PHA</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>54.2%</td>
<td>77.5%</td>
<td></td>
</tr>
<tr>
<td>Mitoses §</td>
<td>Control</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.02</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>(% of total)</td>
<td>PHA</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.78</td>
<td>2.1</td>
<td></td>
</tr>
</tbody>
</table>

* Results are given only for those experiments in which over 99% lymphocytes were obtained.
† Per cent of total cells which stained with Erythrocin B, final concentration 0.06%.
‡ “Large cells” defined as enlarged nuclei with staining characteristics of euchromatin when stained with acetic orcein.
§ Per cent mitotic figures per 1000 cells following mitotic arrest for 2 hours with Velban.
|| Cell counts, because of the clumping and adherence to glass caused by the presence of PHA, are not comparable to control counts (see text).

volume, the cell count in the cultures to which PHA had been added approached that of the control cultures. This drop in cell count was therefore considered to be due to clumping and sticking of PHA-affected cells to glass; such counts were not considered meaningful. By 48 hours, 54 percent of the PHA-stimulated cells had enlarged and the nuclei showed a change from heterochromatin to euchromatin. There was a minimal increase in mitotic rate over that seen in control cultures but, as previously demonstrated, this increase cannot account for any appreciable rise in cell number by 48 hours.

Acid Phosphatase Activity: Biochemical

Cells. The acid beta-glycerophosphatase activity of control lymphocyte cultures showed a slow decrease over time to 76.6 per cent of the initial zero hour activity by 72 hours (Table 2 and Fig. 1). In contrast, cultures to which phytohemagglutinin had been added showed increases averaging 152 per cent of the initial value at 48 hours and 216 per cent of the initial value at 72 hours. Results were expressed as per cent of zero hour values in order to facilitate the comparison of several experiments. Initial zero hour activity (with and without phytohemagglutinin) averaged $163 \pm 23M = \mu$moles P$_1$/10$^7$ cells/hour with a 2.3 per cent average difference between replicate tubes within a single experiment. As seen in Figure 1, the protein content of the
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Table 2
A.—Acid Beta-glycerophosphatase Activity in Cultured Cells*

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>No. of Exp’ts.</th>
<th>Phytohemagglutinin Cells ( \text{molecules Pi/10}^7 \text{ cells/hour} )</th>
<th>Control Cells ( \text{molecules Pi/10}^7 \text{ cells/hour} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>167.3 ± 4.7</td>
<td>159.3 ± 4.8</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>195.0 ± 4.7</td>
<td>161.3 ± 4.8</td>
</tr>
<tr>
<td>24</td>
<td>3</td>
<td>177.7 ± 7.2</td>
<td>137.7 ± 6.2</td>
</tr>
<tr>
<td>48</td>
<td>3</td>
<td>254.0 ± 5.4</td>
<td>136.3 ± 9.0</td>
</tr>
<tr>
<td>72</td>
<td>3</td>
<td>362.0 ± 11.1</td>
<td>122.0 ± 15.1</td>
</tr>
</tbody>
</table>

B.—Acid Phenolphthalein-phosphatase Activity in Cultured Cells and Media*

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>No. of Exp’ts.</th>
<th>Cells</th>
<th>Media</th>
<th>Total</th>
<th>Cells</th>
<th>Media</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>273.0</td>
<td>0</td>
<td>273.0</td>
<td>0</td>
<td>273.0</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>3</td>
<td>198.6</td>
<td>618.6</td>
<td>816.2</td>
<td>185.0</td>
<td>0</td>
<td>185.0</td>
</tr>
<tr>
<td>48</td>
<td>7</td>
<td>483.0</td>
<td>473.0</td>
<td>956.0</td>
<td>204.0</td>
<td>12</td>
<td>216.0</td>
</tr>
<tr>
<td>72</td>
<td>3</td>
<td>768.0</td>
<td>712.0</td>
<td>1480.0</td>
<td>110.0</td>
<td>0</td>
<td>110.0</td>
</tr>
</tbody>
</table>

* Each single experiment consists of replicate culture tubes for both PHA and control.† One experiment.

To facilitate assay of media and to exclude the possibility of a peculiarity of substrate, acid phenolphthaleinphosphatase was also measured. Enzyme activity upon this substrate was very similar to that determined using \( \beta \)-glycerophosphate (Table 2). When calculated as per cent of the initial zero hour value, there was a fall in activity in control cultures as contrasted with an increase in PHA-stimulated cultures which averaged 177 per cent of the initial value at 48 hours and 281 per cent of the initial value at 72 hours. Initial zero hour activity averaged 273. ± 56. \( \mu \text{g. acid phosphatase/10}^7 \text{ cells/hr.} \) with a 6.5 per cent average difference between replicate initial hour tubes within a single experiment.

Media. In addition to increases in enzyme activity within cells, increases in enzyme activity were also found in the supernatant culture media. In order to determine the amount of increased activity, the complete medium was incubated without cells for similar periods and used as a blank, since there was an increase in enzyme activity in the supernatant cultures as contrasted with a decrease in the control cultures which averaged 177 per cent of the initial value at 48 hours and 281 per cent of the initial value at 72 hours. Initial zero hour activity averaged 273. ± 56. \( \mu \text{g. acid phosphatase/10}^7 \text{ cells/hr.} \) with a 6.5 per cent average difference between replicate initial hour tubes within a single experiment.

* Expressed as activity/\( \mu \text{g. protein} \), beta-glycerophosphatase activity was higher in PHA-stimulated cells (667 ± 28) than in control cells (525 ± 72) at 48 hours, a difference significant at the .025 level. Phenolphthalein-diphosphatase activity was also higher in PHA cells (564 ± 74) than in control cells (287 ± 38) at 48 hours, a difference significant at the .01 level.
Fig. 1.—Protein concentration, acid phosphatase activity (betaglycerophosphatase), beta-glucuronidase activity and aryl sulfatase activity of cultured human lymphocytes. Values are expressed as per cent of the activity or content at the onset of culture ("o" hours). For actual values, see text. Culture tubes were removed and the cells were assayed at the indicated hours after onset of culture. Open circles are values for cells with phytohemagglutinin added. Closed triangles are values for cells with no additive.
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Demonstrable acid phosphatase activity in the supplemented fetal calf serum. This activity slowly fell during incubation at 37 C, so that no activity was detected between 24 and 48 hours of incubation. As seen in Table 2, enzyme activity was released into the media removed from PHA-stimulated cells at 24, 48, and 72 hours, and roughly paralleled the enzyme activity remaining within cells.

In early experiments utilizing cultures of lymphocytes contaminated with polymorphonuclear leukocytes (PMNs), a net increase in enzyme activity could not be demonstrated. It was postulated that the presence of hydrolase-rich PMNs (which, however, degenerated during culture) raised the initial values of enzyme activity, but not those observed at 48-72 hours. Therefore experiments were performed with cultures containing varying numbers of PMNs. Plasma from the same individual was separated on two columns; in one, a rapid flow rate yielded 22 per cent PMNs, and in the other the usual flow rate yielded only 5 per cent PMNs. The number of mononuclear cells added to each culture was kept constant. As seen in Table 3, the initial value was indeed twice as high in the cultures containing 22 per cent PMNs compared with the cultures containing 5 per cent PMNs. By 48 hours, at which time the PMNs have degenerated, the absolute activity was the same under both conditions. However, when calculated as per cent of initial zero hour activity, the cultures containing the higher per cent of PMNs showed a fall to 73 per cent, while those containing 5 per cent PMNs showed a rise to 144 per cent of the initial activity. This experiment suggests not only that the presence of hydrolases derived from polymorphonuclear leukocytes could not account for the increased enzyme activity found in PHA-stimulated cultures, but also that their presence would apparently mask any observed net increase.

Histochemical Determination of Acid Phosphatase Activity

Cells grown in the presence of PHA showed a marked increase of acid phosphatase-positive granules compared to control cells cultured without PHA. To quantitate this difference, cells with 3 or more positively staining granules were counted. The results of such experiments are shown in Table 4. Cells were also graded according to the number of granules per cell: 1-2 granules = 1 +; 3-5 granules = 2 +; 6-8 granules = 3 +; and more than 8 granules = 4 +. A typical distribution of cell types at 72 hours is shown in Figure 2. While almost all control cells showed no or insignificant staining for acid phosphatase positive granules, PHA produced significant increases in all classes of positive cells. Typical fields of PHA-stimulated and control cells are shown in Figures 3A and 3B. An occasional single granule can be seen in the control cells, while the stimulated cells contain large numbers of acid phosphatase positive structures. These were distributed in the cytoplasm but frequently tended to aggregate in the perinuclear zone.

Occasionally binucleate cells were observed (Fig. 3C), in which the granules were arranged in linear fashion between the nuclei. When viewed under the phase microscope, these cells appeared to be bounded by a single cell membrane. Whether these cells were in telophase, with granules along
Table 3.—Effect of Contamination with PMN Leukocytes on Acid Phosphatase Activity*  

<table>
<thead>
<tr>
<th>Time (Hrs.)</th>
<th>5% PMN's</th>
<th>22% PMN's t</th>
<th>5% PMN's t</th>
<th>22% PMN's t</th>
<th>5% PMN's t</th>
<th>22% PMN's t</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>36.2</td>
<td>73.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>52.5</td>
<td>54.0</td>
<td>144</td>
<td>73</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean of two experiments.

† Each initial cell inoculum contained the indicated % of polymorphonuclear leukocytes with identical numbers of mononuclear cells.

Table 4.—Acid Phosphatase Staining of Cultured Human Lymphocytes (% of Cells with 3 or more Granules)

<table>
<thead>
<tr>
<th>Exp. #</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>PHA</td>
<td>92.8</td>
<td>75.0</td>
<td>62.0</td>
<td>57.5</td>
<td>44</td>
<td>32</td>
<td>31</td>
<td>30</td>
</tr>
</tbody>
</table>

2. Effect of PPD and SLS at 3 and 5 days  

<table>
<thead>
<tr>
<th>Exp. #</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.7</td>
<td>14.0</td>
<td>5.2</td>
<td>4.0</td>
<td>4.8</td>
<td>1.4</td>
<td>1.7</td>
<td>7.8</td>
</tr>
<tr>
<td>PPD</td>
<td>30.0</td>
<td>42.0</td>
<td>18.0</td>
<td>5.2</td>
<td>—</td>
<td>52.0</td>
<td>18.6</td>
<td>13.2</td>
</tr>
<tr>
<td>SLS</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>8.9</td>
<td>24.0</td>
<td>—</td>
<td>—</td>
<td>35.6</td>
</tr>
</tbody>
</table>

Because of the mitogenic action of antigens on lymphocytes from donors previously sensitized to these antigens, cultures of this sort were also stained for acid phosphatase. Since the height of mitosis under these conditions is reached between 4 and 5 days, cells were stained after 3 and 5 days in culture. As seen in Table 4 and Figure 2, addition of PPD to cells from a tuberculin sensitive donor resulted in the appearance of cells with increased acid phosphatase staining. The nonspecific stimulant to mitosis, streptolysin S (SLS), also caused increases in acid phosphatase-positive granules, as seen in Table 4. SLS is not an antigen and shares with PHA the property of inducing transformation of up to 90 per cent of lymphocytes from all normal donors.

Beta-Glucuronidase Activity

Cells. A second lysosomal hydrolase, beta-glucuronidase, was also assayed biochemically. The enzyme, in PHA-treated cultures, showed no net increase in activity at 48 hours. Indeed, as seen in Figure 1, its activity in such cultures did not differ from control cultures until 72 hours, at which time this could be attributed to the increase in cell number following mitosis. The activity never reached levels higher than that observed at zero hour. Initial zero hour values averaged 8.0 μg. phenolphthalein/10⁷ cells/hour.
HYDROLASE RICH GRANULES IN HUMAN LYMPHOCYTES

Fig. 2.—The effect of PHA and PPD on the number of cells staining for acid phosphatase activity and the intensity of staining. Lymphocytes were cultured for three days and stained by the Gomori method. 1+ = 1–2 acid-phosphate positive granules/cell; 2+ = 3–5 granules per cell; 3+ = 6–8 granules per cell; and 4+ = 8 or more granules.

Media. No beta-glucuronidase activity could be detected in the media removed from the cells, nor was there detectable activity in the sucrose washes.

Aryl Sulfatase Activity

A third lysosomal enzyme, aryl sulfatase, was also assayed. The activity of this enzyme was more variable from experiment to experiment than beta-glucuronidase or acid phosphatase activity. However, as seen in Figure 4, there was a net increase from initial zero hour activity to an average of 182 per cent at 48 hours in cells stimulated by phytohemagglutinin, with a fall in the control cultures to 74 per cent of the initial value at 48 hours. This difference was significant at the 0.025 level.

Fractionation and Release of Sedimentable Enzyme Activity

Because of the dichotomy between the activities of beta-glucuronidase and those of acid phosphatase and aryl sulfatase, an attempt was made to determine if acid phosphatase was associated with membrane-bounded granules. In these experiments only cell suspensions containing over 99 per cent pure lymphocytes were utilized. The average cell lysis (see methods) was 73 per cent.

As seen in Figure 4, 36 per cent of the total recovered acid phosphatase activity, and 44 per cent of the total recovered beta-glucuronidase activity sedimented with the "nuclear" fraction (NM). This represented activity in un-
Fig. 3A.—Control lymphocytes cultured for 3 days without the addition of phytohemagglutinin and stained for acid phosphatase (Gomori method). Nuclei are lightly counterstained.

Fig. 3B.—Lymphocytes cultured for 3 days with the addition of phytohemagglutinin and stained for acid phosphatase, showing both an increase in the number of cells containing acid phosphatase positive granules and in the number of granules in each cell.
broken cells as well as some from the large granule (mitochondrial) fraction, because of the speed of centrifugation used. The higher speed of centrifugation was chosen so as to eliminate contamination of the 15,000 g pellet with unbroken cells. In the 15,000 g, or granular (G), pellet was found 44 per cent of the acid phosphatase activity and 36 per cent of the \( \beta \)-glucuronidase activity. Twenty per cent of both enzyme activities was found in the supernatant fraction (S) and represented either nonlysosomal acid phosphatase and \( \beta \)-glucuronidase, or enzyme released during homogenization. The relative specific activity of the 15,000 g fraction (G) showed a twofold increase over the total homogenate (T).

To determine whether this sedimentable enzyme activity could be shown to be associated with membrane-bounded granules, fractions were incubated with and without 10\(^{4}\) M lysolecithin for 30–45 minutes at 24°C, centrifuged at 15,000 g for 20 minutes, and the supernates, pellets, and aliquots of the unspun fractions were assayed for enzyme activity. Under these conditions, 64 per cent of total “nuclear” acid phosphatase and 60 per cent of total “nuclear” \( \beta \)-glucuronidase (NM) were released into the supernate. In contrast, 92 per cent of the acid phosphatase and 91 per cent of the \( \beta \)-glucuronidase were released from the granular fraction (G) into the supernate. Enzyme activities remaining in the pellets were reciprocal to those recovered in the supernates. Lysolecithin did not affect enzyme activity in the cell supernatant fractions (S).

The Effect of Chloroquine on Acid Phosphatase Activity of Human Lymphocytes

It has been suggested that lysosomes may become labilized during the early
Fig. 4.—A: Relative specific activity of fractions obtained by differential centrifugation of homogenates of lymphocytes previously cultured with PHA for 48 hours. See text for details of fractionation. T = total homogenate; NM = 1000 g × 10'; G = 15,000 × g-20'; S = supernate.

B: Percentage of the total recovered activity present in each of the subcellular fractions. Each fraction was also treated with a membrane disruptive agent (Lysolecithin, 10^{-3}M). The crosshatched plus the striped areas represent the enzyme activity that could no longer be sedimented at 15,000 g—i.e., released after treatment with lysolecithin. The striped area represents enzyme activity which could no longer be sedimented in samples incubated without lysolecithin—i.e., released by spontaneous rupture under the conditions of incubation. Acid phosphatase was assayed with phenolphthalein diphosphate as substrate.

Chloroquine is an agent which is known both to stabilize lysosomes \(^{21}\) and to inhibit stimulation of cultured lymphocytes.\(^ {22}\) The effects of chloroquine on development of acid phosphatase activity in PHA-stimulated cells was studied. Chloroquine diphosphate was
HYDROLASE RICH GRANULES IN HUMAN LYMPHOCYTES

Table 5.—Effect of Chloroquine on Acid Phosphatase Staining and Large Cell Formation at 72 Hours

<table>
<thead>
<tr>
<th>Expt. #</th>
<th>Control</th>
<th>Control + C1</th>
<th>PHA</th>
<th>PHA + C1</th>
<th>PHA + C1t</th>
<th>Control + C1*</th>
<th>PHA</th>
<th>PHA + C1</th>
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<tr>
<td>1.</td>
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<td>0</td>
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<td>—</td>
<td>12.8</td>
<td>—</td>
<td>81.0</td>
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<tr>
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<td>64</td>
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<td>30</td>
<td>2.2</td>
<td>10.0</td>
<td>78.4</td>
</tr>
<tr>
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<td>30.5</td>
<td>7.3</td>
<td>—</td>
<td>5.2</td>
<td>—</td>
<td>78.0</td>
</tr>
</tbody>
</table>

* C1 = chloroquine (10^{-4} M) added at onset of experiment.
† C1 = chloroquine (10^{-8} M) added 4 hours before termination of experiment.

added in a final concentration of 10^{-5} M before the addition of PHA (C1).

Cells were harvested at 72 hours and stained for acid phosphatase, while replicate tubes were studied for large cell formation and mitotic rate. As seen in Table 5, chloroquine inhibited both acid phosphatase staining and cell stimulation. To determine if this inhibition of staining was due to decreased permeability of the lysosomal membrane to substrate (which appeared unlikely in view of the prolonged fixation time), chloroquine was also added 2 hours before harvesting (C1). Although there was diminution of staining to half that seen in cultures exposed to PHA alone, the number of granules was still greater than that observed in PHA-stimulated cultures to which chloroquine had been added at the onset (C1). This effect of chloroquine does not represent inhibition of free enzyme activity. Therefore, these experiments suggested that chloroquine both stabilized existent lysosomes, with reference to their permeability produced by staining and fixation, and that it prevented PHA-induced stimulation.

DISCUSSION

The experiments reported above have confirmed and extended our preliminary report of increased acid phosphatase activity in PHA-stimulated lymphocytes. Biochemical assays have demonstrated a marked net increase in two lysosomal acid hydrolases: acid phosphatase activity (determined both with β-glycerophosphate and phenolphthalein phosphate as substrates) and aryl sulfatase. It is as yet unclear whether the two acid phosphatases are identical; certainly both are lysosomal, and indeed the β-glycerophosphatase is almost exclusively lysosomal, at least in liver. Increases in acid hydrolase activity of PHA-stimulated cells were accompanied by a relatively smaller increase in total cellular protein. However, the activity of another lysosomal hydrolase, β-glucuronidase, did not increase until there was an increase in the number of cells per culture. Because of this dissociation between the activities of the two hydrolases, the localization and latency of the acid phosphatase was investigated. The greater portion of the enzyme activity was shown to be in the granular fraction of the cell homogenates, and bounded, or "latent," in that it was releasable by lysolecithin, a membrane-disruptive agent. The distribution of enzyme activity among "nuclear," "large granule," and "super-
natant" fractions of PHA-stimulated lymphocyte homogenates was comparable to the distribution seen in fractions of liver homogenates and human polymorphonuclear leukocytes, tissues from which lysosomes have previously been isolated.23,24

In cultures stimulated by PHA, the increase in acid phosphatase activity per cell was further documented by histochemical studies, which confirm and quantitate our earlier report.7 Allison and Mallucci25 and Parker26 have also demonstrated an increase in acid phosphatase-staining granules prior to PHA-induced mitosis of lymphocytes.

Coincident with stimulation, lymphocytes undergo many other, possibly unrelated, metabolic changes which introduce difficulties into the experimental system. Because lymphocytes clump in the presence of PHA, it is difficult to obtain comparable cell counts in the treated and untreated populations. The apparent drop in cell count (as determined by conventional means) in PHA-treated cultures, might reflect a toxic effect of phytohemagglutinin. Cell injury may thus have been the stimulus which influenced the increase in lysosomal hydrolases. However, two pieces of evidence suggest that PHA induced only an apparent, rather than a real, change in cell number. First, the cell count in the PHA-treated cultures could be raised to values approaching that of the control cultures by measures which eliminated the cells' clumping and sticking to glass. Second, the rapid drop in cell count which was observed during the first 4 hours after addition of PHA to the cultures was not preceded by an increase in nonviable cells, nor was there a concomitant decrease in the total protein.

On the other hand, the possibility could not be eliminated that there was a one and a half-fold increase in total cell number at 48 hours in PHA-stimulated cultures. Such an increase could account for the increase in enzyme activity. However, earlier studies19 of cell kinetics indicate that at 36 hours approximately only 2.5 per cent of the cells are synthesizing DNA. The length of the S (or DNA synthetic) period which precedes mitosis has been estimated27 at a minimum of 12 hours during the first cell cycle. Therefore, an appreciable increase in cell number at 48 hours is very unlikely. In addition, the increase in number of acid phosphatase-staining granules within individual cells, seen histochemically, indicates that there has been a net increase in enzyme activity per cell. The lack of an increase in β-glucuronidase activity until 72 hours would also indicate that there was no essential increase in the number of cells in PHA-stimulated cultures at 48 hours.

It has been postulated that the observed increase in acid phosphatase activity represents nonlysosomal enzyme activity and "relates to the transport across the cell membranes of nutrients necessary during cell growth."28 Since acid phosphatase was "identified occasionally in granules but more consistently in small vesicles throughout the cytoplasm and particularly in the Golgi complex,"29 the increase in acid phosphatase was thought to reflect an increase in Golgi activity. Coulson6 has previously demonstrated increased activity of the Golgi complex in stimulated lymphocytes. Some of the increased acid phosphatase activity may indeed be localized to the Golgi complex.
However, recent studies of the mechanism of formation of lysosomes in mouse phagocytes indicate that both the activity of the Golgi complex and lysosome formation may be related to endocytosis, and that under these conditions Golgi vesicles may transfer hydrolases to endocytic vacuoles or other "secondary lysosomes." Parker has recently demonstrated an increase of lysosomes in PHA-stimulated lymphocytes by electron microscopy. The increase in aryl sulfatase activity, an enzyme not biochemically localized to other organelles but present in lysosomes, would also indicate that PHA induces an actual increase in the number of these organelles. In addition, data obtained from cell fractionation studies indicated that only 20 per cent of acid phosphatase activity could be recovered in the 15,000 g supernate, where one would expect to find smaller vesicles. However, 44 per cent of the activity was associated with the large granules, fractions which in other leukocytes are lysosome-rich. These results would suggest the presence of lysosomes in PHA-stimulated cells.

The significance of these changes in hydrolytic enzyme activity is not clear at present. It is unlikely that the amounts or activities of all hydrolytic enzymes become increased, since β-glucuronidase activity remained constant. It has been suggested that lysosomes may develop in order to facilitate breakdown of the increased glycogen seen in these cells prior to DNA synthesis. Mazia, extrapolating from saltatory motion of particles in lower organisms, has previously postulated that concentration at the nuclear membrane of lysosomes, with subsequent release of their hydrolytic enzymes, might play a role in the dissolution of the nuclear membrane. Adams has shown a cyclic variation of 3 lysosomal hydrolases with the cell cycle in regenerating rat liver. Activity of hydrolases reached a peak prior to the mitotic wave, fell during division, and rose again during the second cycle. Recently, several electron microscopic studies have provided morphologic evidence of the formation of lysosomes before the second wave of mitosis in regenerating rat liver. Our observations that lysosomes became clustered about the nuclear membrane (or nuclear hof area) would suggest the possibility that lysosomal enzymes play a role in the intensive remodeling processes of cell division.

To determine whether lysosomes arose only as a response to phytohemagglutinin, or whether they were involved in the effect of other mitogenic agents, the nonspecific mitogen, SLS, and a more specific agent, PPD, were added to cultures. With PPD, the cells were obtained from naturally sensitized donors. Histochemical studies confirmed the increase in acid phosphatase activity with both of these agents. Biochemical assays were not performed on antigen-stimulated cultures for two reasons: With antigen stimulation, a relatively small proportion of the cells respond (5–35 per cent) so that a 150 per cent increase in the hydrolase activity of this population would be undetectable by the gross technics we have employed. In addition, at the time of maximal antigen stimulation (3–5 days), it was not possible to maintain an unchanging control population as judged by variations in total cell number. Diengdoh and Turk have demonstrated that a similar increase in acid phosphatase positive granules occurs in sensitized lymphocytes in vivo following antigenic chal-
lene. This enzyme activity behaved as if it were membrane-bounded in that the positivity of staining could be abolished by incubation with a membrane-disruptive agent.

When chloroquine was added to cultures of stimulated lymphocytes, it not only inhibited stimulation but also prevented increases in the number of acid phosphatase-staining granules. This would suggest that the increase in acid phosphatase positive granules is a result of the stimulation of lymphocytes and not an independent event associated with the addition of agents used to produce such stimulation. It is entirely possible that chloroquine exerted these effects not because it stabilized lysosomes, but by virtue of its capacity to bind to DNA in vitro and to inhibit DNA and RNA polymerase. However, on the basis of other, albeit indirect, evidence it has been suggested that an early event during lymphocyte stimulation may be the labilization of lysosomes or other membrane-bounded structures. Cooper and Rubin have demonstrated that within 30 minutes of the addition of PHA there is a rapid breakdown of existing RNA, simultaneous with rapid uridine incorporation into new RNA. Conceivably this effect may reflect the consequences of release of lysosomal RNase. Two substances which mimic PHA in causing stimulation of virtually all lymphocytes are streptolysin S and staphylococcal exotoxins; each of these has been shown to release enzymes from lysosomes. In contrast, chloroquine, prednisolone, and chlorpromazine, substances which stabilize the lysosomal membrane, inhibit the action of PHA. Finally, Allison has provided histochemical evidence for increased permeability of lysosomal membranes early in the course of lymphocyte stimulation by PHA.

It is of interest that a third lysosomal enzyme, β-glucuronidase, did not increase, but followed the pattern found in nonstimulated lymphocytes. This discrepancy indicates either that lymphocyte lysosomes are quite heterogeneous, or that premitotic activity provokes the production of "incomplete" organelles. Conceivably such heterogeneity reflects a difference in the origin of these lysosomes—that is, from the Golgi apparatus vs. the endoplasmic reticulum, as discussed by several authors.

SUMMARY

Purified human peripheral blood lymphocytes have been shown to develop acid hydrolase-rich granules between 24 and 48 hours after stimulation by phytohemagglutinin, prior to mitosis. This increase has been measured biochemically as a net increase in total activity of the lysosomal enzymes: acid β-glycerophosphatase, acid phenolphthalein phosphatase, and aryl sulfatase. The subcellular localization of acid hydrolytic enzyme activities has been investigated, and they have been shown to be concentrated in a large granule fraction of sucrose homogenates and to behave as if they were membrane-bounded, in that their activity could be released by lysolecithin. It has also been demonstrated by histochemical technics that stimulation of lymphocytes by antigen (PPD) and by streptolysin S, as well as by phytohemagglutinin, produced an increase in acid phosphatase activity. Chloroquine, an inhibitor of the response to phytohemagglutinin, has also been shown to inhibit the development of acid phosphatase activity. These results are interpreted to
suggest that both specific and nonspecific stimulants of lymphocytes induce lysosome-like structures in premitotic cells.

**SUMMARIO IN INTERLINGUA**

Purificate lymphocytos ab human sanguine peripheric disveloppava granulos nc in hydrolase acide inter 24 e 48 horas post stimulation con phytohemagglutinina ante le tempore del mitose. Iste augmento esseva mesurate biochimicamente como un augmento nette in le activitate total del enzymes lysosomal: /3-glycerophosphatase, phosphatase de phenolphthaleina acide, e sulfatase arylic. Le localisation subcellular de activitate de enzymes hydrolytic acide esseva investigate. Esseva demonstrate que illo es concentra in un fraction macro-granular de homogenatos de sucrosa e que illo se comporta como si illo esseva inherent in entitates a inveloppe membranose, proque illo poteva esser liberate per lysolecithina. Esseva etiam demonstrate per medios histochimic que le stimulation de lymphocytos per antigeno (PPD) e per streptolysina S si ben como per phytohemagglutinina produceva un augmento in le activitate de phosphatase acide. Chloroquina, un inhibitor del responsa a phytohemagglutinina, se ha etiam monstrate capace a inhibir le disveloppamento de activitate de phosphatase acide. Iste resultatos es interpretate de maniera a suggestionar que tanto specific como etiam nonspecific stimulantes de lymphocytos induce structuras lysosomoide in cellulas premitotic.

**REFERENCES**

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Appearance of Hydrolase Rich Granules in Human Lymphocytes Induced by Phytohemagglutinin and Antigens

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