THE EFFECT OF PHYTOHEMAGGLUTININ ON RIBONUCLEIC ACID SYNTHESIS AND HISTONE ACETYLATION IN EQUINE LEUKOCYTES

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It is well known that peripheral lymphocytes rarely divide in tissue culture; indeed, they have usually been considered to be the end cell in the pathway of differentiation. However, in the presence of certain mitogenic agents, of which phytohemagglutinin (PHA) is the best known example, a striking transformation occurs; the cells increase their over-all metabolic activity, enlarge, and divide. This response of the small lymphocyte, widely studied since its discovery in 1960 (1, 2), is now known to involve an increased synthesis of ribonucleic acids (3–5). It has also been observed that histone metabolism is altered very early in the process of gene activation for RNA synthesis (5). The acetylation of arginine-rich histones, in particular, is increased very soon after exposure of the cells to the mitogenic agent.

Relatively little attention has been paid to the effect of PHA on other cell types. Recently Andrew and Gabourel (6) described cytotoxic effects of PHA on rapidly growing cells in vitro and in vivo. On the other hand, Ioachin (7), found increased mitotic indexes in cell lines in tissue culture under the effect of PHA; Gamble also observed a stimulatory response in all cell types in mouse spleen in vivo (8). Ameba growth was also stimulated by PHA (9).

In polymorphonuclear leukocytes, only vacuolization has been found after some hours in tissue culture in the presence of PHA (10, 11), and Razari found fluorescence in the polymorphonuclear cell cytoplasm as soon as 15 min after exposure to fluorescent PHA (12).

In a previous publication (5), the correlation between RNA synthesis and histone acetylation was studied in lymphocytes stimulated by PHA. We also observed that PHA inhibited both processes in equine granulocytes. A detailed account of these effects in equine lymphocytes and granulocytes (with special reference to RNA synthesis and histone acetylation) will be reported here.

MATERIAL AND METHODS

Equine citrated blood was used in all the experiments. The preparation of cell suspensions, incubation procedures, and chemical analyses were described elsewhere (5). Some modifications of the previous methods were introduced in the separation of white

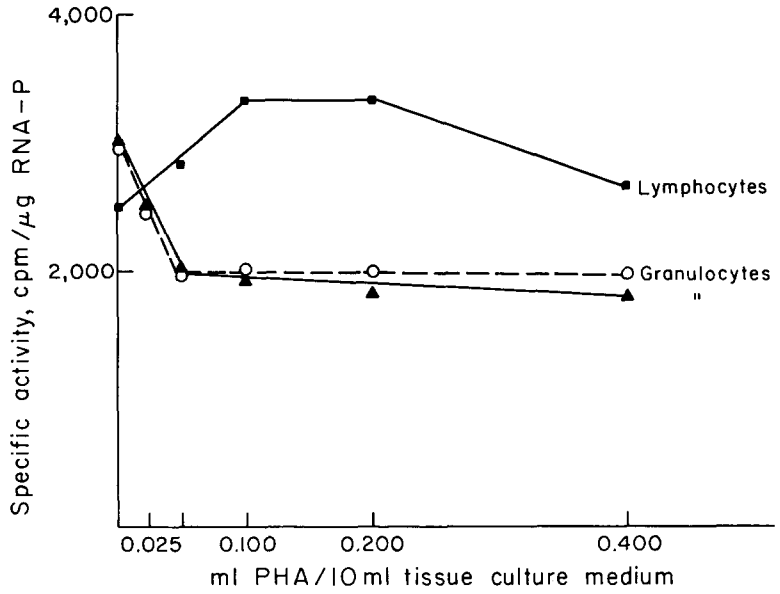

FIGURE 1 Effect of increasing concentrations of phytohemagglutinin on RNA synthesis by equine granulocytes and lymphocytes in culture. The specific activity of the total RNA after 60-min incubation with uridine-2-^{14}C (specific activity 30 mc/mmole; 0.5 μc per ml) is plotted against the PHA concentration of the medium.

cells from the plasma. The centrifugation of plasma and white cells was carried out at 600 *g* instead of the 1,000 *g* used before. This was done to avoid clumping of white cells. The cell pellets were resuspended in Eagle's Minimal Essential Medium (MEM) with 1 mg/ml heparin and centrifuged at 100 *g* for 2 min. The supernatant contains the lymphocytes; the pellet, mainly polymorphonuclear cells. This last centrifugation can be repeated to obtain almost pure polymorphonuclear leukocyte suspensions (with only 4–5% contamination by mononuclear cells). (Contamination with mononuclear cells above 10% would invalidate the results since the lymphocytes are rapidly stimulated by PHA.) The same tissue culture medium (MEM with 20% newborn calf serum) was used as in reference 5. The final cell concentration varied from 2×10^6 to 5×10^6 cells/ml. These were the cell concentrations found to have optimal activity in uridine-2-^{14}C uptake.

RESULTS

Effect of Different Concentrations of PHA on RNA Synthesis

Equine polymorphonuclear leukocyte suspensions were incubated for 60 min in the presence of PHA and uridine-2-^{14}C. Various PHA concentrations were tested. (The PHA used was PHA preparation "P" (Difco) dissolved in 5 ml of distilled water.) In Fig. 1 are shown the results of such experiments. Two polymorphonuclear leukocyte suspensions prepared from different animals showed the same degree of inhibition of uridine uptake into RNA. An entirely different response is shown by a sample of equine lymphocytes tested under the same conditions; RNA synthesis in the lymphocyte is stimulated, as has been reported earlier by many workers (3–5). RNA synthesis in polymorphonuclear cells is inhibited by Actinomycin D (Fig. 2), indicating its DNA-dependence.

Effect of PHA on RNA Synthesis and Histone Acetylation

Since we had found a correlation between RNA synthesis and histone acetylation in lymphocytes (5), experiments were designed to see which of these processes is first inhibited when PHA is added to polymorphonuclear cells. "Pulse-labeling" experiments using orotic acid-6-^{14}C or acetate-2-^{14}C as precursors were carried out. Cell suspensions exposed to PHA for different lengths of time were tested for their capacity to synthesize RNA or to acetylate histones in a short, fixed time

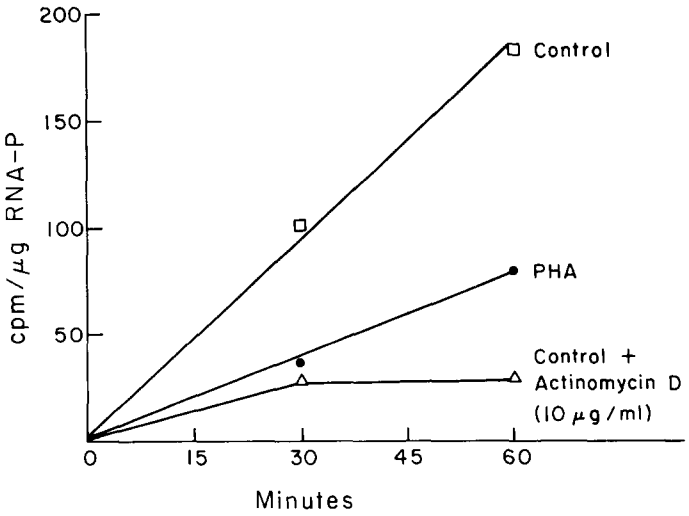

FIGURE 2 Time course of orotic acid-6-^{14}C (Sp.Act. 4.9 mc/mmole; 5 μc per ml) uptake into the total RNA of equine granulocytes. The uppermost line shows the rate of RNA synthesis in control cells, and the middle line shows the corresponding rate in cells exposed to PHA. The inhibitory effect of Actinomycin D on RNA synthesis in control cells is shown in the bottom curve.

interval (15 min). The results are shown in Fig. 3. It should be noted that freshly isolated cells placed in culture under these conditions usually exhibit a gradual increase in their RNA synthetic capacity. This is evident in the increased uptake of ^{14}C-labeled orotic acid measured as described over 15-min intervals, taking equal aliquots of the cell suspension at successive time points after initiation of the culture. (An increased capacity for RNA synthesis by phagocytosing granulocytes in culture has been reported earlier by Cline (13.)) In this respect, the kinetics of orotic acid incorporation into RNA of the control granulocytes resemble those observed in lymphocytes after stimulation (5).

In lymphocytes the acetylation of arginine-rich histones was observed to precede RNA synthesis (5). There is some suggestion that this is true in granulocyte cultures as well, since the increased capacity for histone acetylation is evident within 15 min in the control cells.

PHA Inhibits both RNA Synthesis and Histone Acetylation in Granulocytes

The inhibition of acetate incorporation by PHA seems slightly greater than the inhibition of orotic acid uptake at the first time point tested (15 min).

Puromycin was used as an inhibitor of protein synthesis to see whether the acetate-^{14}C was incorporated into the histone as acetyl or as amino acid (i.e., as aspartate or glutamate derived from ^{14}C-acetate). As can be seen in Table I, puromycin does not inhibit acetate incorporation into the histones. This rules out the possibility that appreciable amounts of acetate-^{14}C were incorporated as aspartic or glutamic acids in the protein. The same puromycin concentration (10 μg/ml) can be seen to inhibit alanine uptake into the histones and into residual nuclear proteins. It is worth mentioning that PHA treatment also decreased alanine incorporation into both classes of nuclear protein in granulocytes, while the opposite effects were observed in lymphocytes treated in this way (5).

Effect of PHA "Turnover" of Acetyl Groups and Amino Acids Previously Incorporated into Nuclear Proteins

It might be objected that granulocytes treated with PHA in this way are damaged and, therefore, suffer a wide variety of metabolic derangements.

A more significant test is to study the effect of the addition of PHA on the stability of acetyl groups *previously incorporated* into the histones of *undamaged* cells (i.e. cells incubated with acetate-^{14}C under control conditions). Experiments on patterns of histone acetylation in liver cells have

TABLE I

Incorporation of Acetate-2-^{14}C and Alanine-^{3}H into Nuclear Proteins of Control and PHA-Treated Cells

Precursor	Concentration	Uptake into histones* of: Control cells	PHA-treated cells	Puromycin-treated control	Uptake into "residual protein" ‡ of: Control cells	PHA-treated cells	Puromycin-treated control
Acetate-2-^{14}C§	1 μc/ml	0.300	0.160				
" "	2 μc/ml	0.450	0.210				
" "	"	0.420	0.310	0.400			
Alanine-^{3}H‖	20 μc/ml	0.080	0.043	0.043	3.150	1.720	1.230

* $\mu\mu$ moles per 100 $\mu\mu$ moles histone.
‡ Counts per minute per milligram protein.
§ Specific activity 25.5 mc/mmole.
‖ Specific activity 332 mc/mmole.

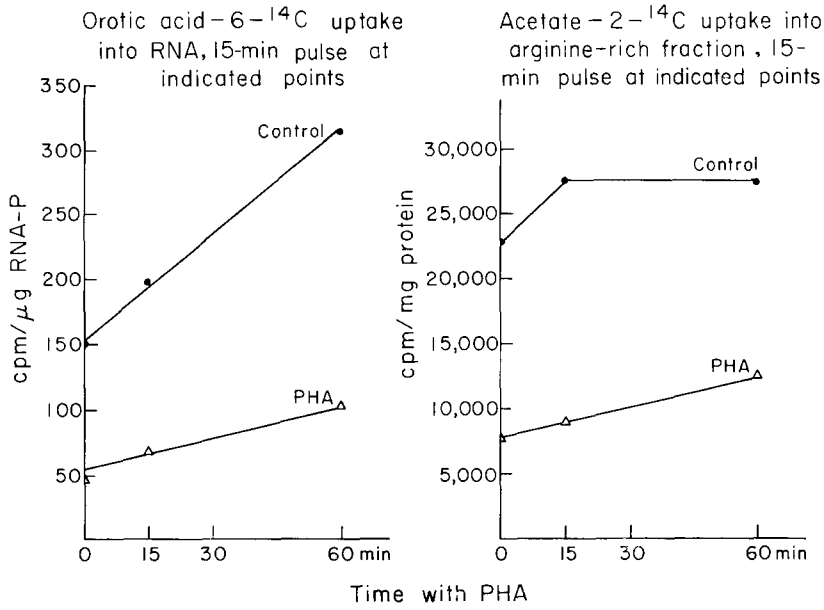

FIGURE 3 Comparison of RNA synthesis and histone acetylation in granulocytes with and without phytohemagglutinin. A uniform cell culture was divided into two portions, one serving as a control and the other receiving PHA. Aliquots were withdrawn at the time points indicated and incubated for a fixed interval (15 min) in the presence of orotic acid-6-^{14}C (Sp.Act. 4.9 mc/mmole; 5 μc/ml) or Na Acetate-2-^{14}C (Sp.Act. 25.5 mc/mmole; 2 μc per ml). The radioactivity of the RNA or histone is plotted against the time of exposure to PHA.

indicated that histone acetyl groups are in constant exchange or "turnover," and that such turnover is minimal at times of gene activation for RNA synthesis (B. G. T. Pogo, V. G. Allfrey, and A. E. Mirsky. Manuscript in preparation). It was thus expected that acetyl group "turnover" would be increased in cells, such as granulocytes exposed to PHA, in which RNA synthesis was inhibited.

Polymorphonuclear leukocytes were preincubated with acetate-2-^{14}C for 30 min under control conditions, then washed three times with nonradioactive medium, and divided in two aliquots:

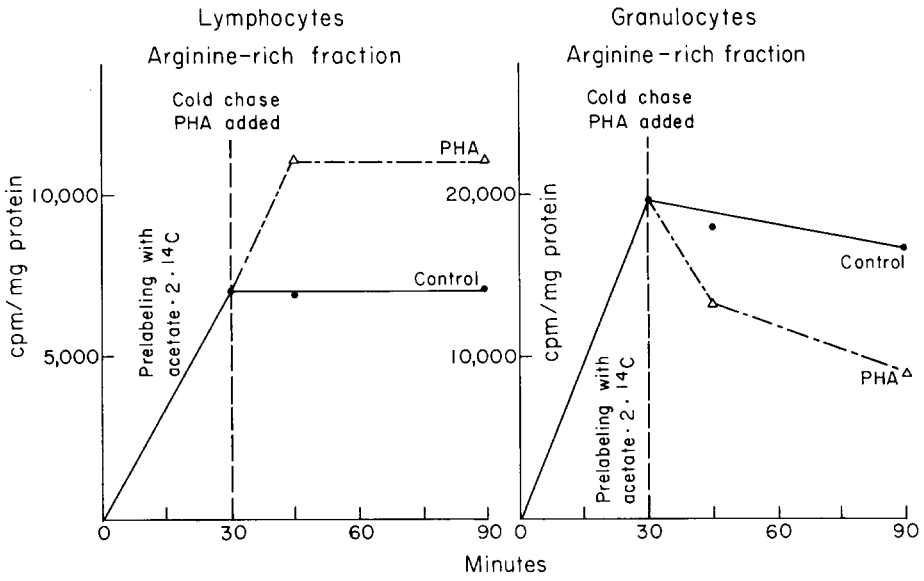

FIGURE 4 A comparison of acetate-^{14}C uptake and retention in histones of lymphocytes and granulocytes exposed to phytohemagglutinin after prelabeling under control conditions. The cells were preincubated for 30 min in the presence of Na Acetate-2-^{14}C (Sp.Act. 25.5 mc/mmole; 2 μc per ml), washed, and resuspended in nonradioactive medium. Phytohemagglutinin was added to the cell suspensions at this time. The specific activity of the arginine-rich histone fraction is plotted as a function of time during the "cold chase."

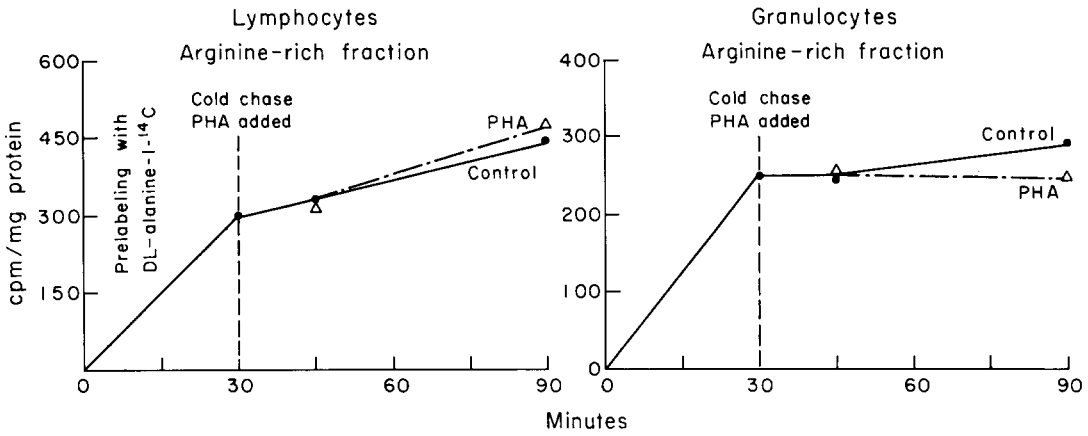

FIGURE 5 A comparison of alanine-1-^{14}C uptake and retention in histones of lymphocytes and granulocytes exposed to phytohemagglutinin after prelabeling under control conditions. The cells were preincubated for 30 min in the presence of DL-alanine-1-^{14}C (Sp.Act. 8.1 mc/mmole; 2 μc per ml), washed, and resuspended in nonradioactive medium. Phytohemagglutinin was added to the cell suspensions at this time. The specific activity of the arginine-rich histone fraction is plotted as a function of time during the cold chase.

one was incubated with PHA, the other used as control. Samples were taken at 15 and 60 min. A similar experiment was carried out with DL-alanine-1-^{14}C as a precursor. As can be seen in Figs. 4 and 5, the loss of previously incorporated acetyl groups is higher in the granulocytes treated with PHA than in the corresponding controls. For comparison, the same type of experiment was

carried out with lymphocytes. In these cells, PHA increased the amount of acetyl groups incorporated into the histones over the same time period. (The increase in specific activity of the lymphocyte histones during what should have been a "cold chase" probably represents the continuing mobilization of available acetyl-^{14}C groups from the "pool.")

It is important to note that the stability of previously incorporated amino acid does not show any significant change in lymphocytes or granulocytes treated with PHA under these conditions (Fig. 5). This finding makes it very probable that the loss of acetate-^{14}C label represents a loss of acetyl groups and is not due to a loss of histone proteins. It should also be stressed that the data given represent the specific activity of the ^{14}C-acetylated histones after their isolation and purification by electrophoresis (5). It is clear that the histones in PHA-treated cells have lost acetyl groups which were previously incorporated under normal conditions. Though this hardly proves that the loss of acetyl groups is *causally* related to the impaired capacity for RNA synthesis, our data indicate that histone deacetylation is at least a very sensitive and early measure of impaired cell function. In short, under conditions in which the synthetic capacities of the granulocyte are restricted, there is a correlation between the inhibition of RNA synthesis and a reduction in histone acetylation.

DISCUSSION

Equine granulocytes after being isolated from the blood and transferred to cell culture conditions showed a depressive response to PHA. These cells are not able to divide, but undergo lysis after some hours in tissue culture; nevertheless, they are able to synthesize RNA and proteins when freshly isolated (13).

The rapid effect of PHA on granulocytes, evident in the inhibition of RNA synthesis and histone acetylation within 15 min, is also evident in the agglutination of the cell suspension, an effect which suggests involvement of the cell membranes. Experiments now in progress indicate that membrane effects do play a role in response to PHA by both types of leukocytes (B. G. T. Pogo. To be published).

The remarkable difference in the behavior of the preincorporated acetyl groups in the two types of cells can be correlated with the inhibitory or stimulatory response in RNA synthesis. It is interesting to note that only the acetyl groups "turn over" rapidly in the inhibited cells, and not the amino acids previously incorporated into the histone molecule. It appears that when the cell loses its capacity for RNA synthesis it also discards acetyl groups from the histones. The significance of the changes in histone structure and their relationship to the capacity of the DNA to serve as a template for RNA synthesis have already been discussed (5).

SUMMARY

Equine granulocytes exposed to phytohemagglutinin show a decreased capacity for RNA synthesis, unlike lymphocytes in which PHA stimulates RNA synthesis. The inhibited granulocytes also show a decrease in the rate of acetylation of arginine-rich histones. If the histones are acetylated under control conditions and the cells subsequently exposed to PHA, the acetyl groups are rapidly discarded, while amino acids incorporated into the histone remain stable. Though these findings do not constitute a proof of a causal relationship between histone deacetylation and the inhibition of RNA synthesis, they do offer further support for the view that the functional state of the chromatin is reflected in patterns of histone acetylation.

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