Studies on Inhibition of Human Lymphocyte Blastogenesis by Hydrocortisone in vitro

I. Effect of Hydrocortisone on Lymphocyte Response to Phytohemagglutinin

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The effect of adrenal corticosteroids on the immune system has been extensively investigated during the past several decades (see Awaya 1975[1]). While this work has provided a rational basis for the clinical use of corticosteroids during transplantation surgery and in the treatment of allergic diseases, the precise mechanism of the action of the corticosteroids on the immune system in humans remains unclear.

Nowell (1961)[2] reported the suppressive effect of prednisolone on the transformation of lymphocytes in PHA culture. This was confirmed by several authors[3-10]. Although the mechanism of this phenomenon also is not fully understood, it is of great interest to observe the early and late changes which occur in lymphocytes in PHA culture, by the addition of hydrocortisone, in attempting to interpret some of the complex in vivo processes such as the suppressive effect of the hormone on homograft response.

The present study was designed to examine the effect of hydrocortisone on the human lymphocyte in PHA culture, with special reference to the selection of the quantity of the dose of hydrocortisone and the time for its administration. In addition, the effect of the hormone on some early events in PHA cultured lymphocytes—e.g. nuclear histone changes and DNA synthesis—also was investigated.

MATERIALS AND METHODS

Test materials: One vial of dry PHA powder (PHA-P, Difco, U.S.A.) was dissolved in 5 ml of sterile distilled water. Various concentrations of PHA solution were made by diluting with sterile physiological saline. Hydrocortisone (Fluka AG, Buchs SG, Swizerland) was dissolved in 95%

ethanol at a concentration of 15 mg/ml and diluted with sterile distilled water to give final concentration (10 μg/ml). When necessary, further dilutions were made with Hanks balanced salt solution (Difco).

Lymphocyte separation: Lymphocytes were separated according to a modification of the method of Böyum[11]. Ten milliliters of human venous blood from healthy adult male volunteers were drawn aseptically into a syringe containing 200 units of heparin (heparin natrium, Takeda). The heparinized blood was diluted with 20 ml of sterile physiological saline. Then 6 ml of the blood solution were carefully layered on the top of 3 ml of Ficoll-Isopaque solution (Lymphoprep, Nyeggard & Co. As., Oslo) which was prepared in a 10 ml centrifuged tube. The tube was centrifuged at 1500 rpm for 30 minutes. The lymphocyte rich fraction (more than 90% in population) was gently removed and the cells were then washed three times; each washing was with 5 ml of the chilled Hanks solution and was followed by centrifugation of the solution at 700 rpm for 10 minutes. The washed cells were finaly resuspended at a concentration of 10^6 cells/ml in the TC-199 medium (Chiba) containing 20% calf serum (Chiba). The viability of the cells in the suspension was determined by a dye-exclusion technique using Trypan blue as described by McLimans et al.[12]

Lymphocyte culture: One milliliter of cell suspension was added to a 14×100 mm sterile test tube with Morton stainless steel cap. The cells were cultured in a 95% air-5% CO_2 humid atmosphere at 37°C for 72 hours. The PHA and hydrocortisone to be tested were added to the 1 ml of cell suspension in a volume of 0.01-0.05 ml. Unless otherwise noted, the cells were exposed to PHA (10 μg/ml) and hydrocortisone (100 μg/ml) simultaneously. These dose levels were chosen because these concentrations were most appropriate for the lymphocyte transformation and its inhibition as described below.

Harvesting of culture: At appropriate times after initiating the culture, the cells from each test tube were washed twice with 5 ml of the Hanks solution and then resuspended in 1 ml of Haemaccel (Hoechst). After centrifugation of the cell suspension at 1000 rpm for 10 minutes, the cell pellets were smeared onto slides for estimating the degree of lymphocyte transformation. The smear were stained with Wright's stain and on each slide more than 1000 lymphoid cells were counted within one-third of the smear area. Care was taken to distinguish between blastoid cells and large macrophage-like cells, or intact and degenerated lymphocytes. Lymphocytes with increased cytoplasm, but little or no nuclear changes were not classified as blastoid cells. The percentage of blastoid cells from counted cells was evaluated as the index of blasto-

genesis.

Quantitative estimation of DNA and nuclear histone in cultured lymphocytes: Smears of lymphocytes in the cultures containing PHA and/or hydrocortisone were made on one-half of a cover glass (25× 50 mm, No. 1) and smears of lymphocytes in the control culture containing neither PHA nor hydrocortisone on the other half.

The DNA of cultured lymphocytes was demonstrated by the Feulgen reaction. The smears were fixed in Carnoy's fluid for 30 minutes and hydrolyzed with 5N HCl at room temperature for 15 minutes according to the method described by Tomonaga et al.[13] Subsequently, the smears were stained with Schiff's reagent for 3 hours by the method of Seno and Utsumi[14] and washed in 3 changes of SO_2 water. After a final rinse in running tap water the slides were mounted in immersion oil. Throughout these procedures the experimental and control smears on a slide were exposed to the same conditions. Therefore, the intensity of the staining in both smears could be directly compared. The amount of Feulgen-stained DNA was measured at 560 mμ with a microspectrophotometer (Olympus MSP-AIV). In each smear, 100 cells were measured and a mean value was calculated according to the method of Tomonaga et al.[15]

Staining with alkaline fast green was used to determine the relative levels of nuclear histone in the cultured lymphocytes. As described above, both the experimental and control smears were prepared on a single cover glass. The smears were fixed for 10 minutes in 10% neutral buffered formalin. Hydrolysis with 5% trichloroacetic acid was performed at 90°C for 15 minutes according to the method of Alfert and Geschwind[16]. The slides were stained in a 0.1% solution of fast green FCF (Chroma), buffered at pH 8.0 for 30 minutes at room temperature. Finally, the slides were washed in distilled water, dried and mounted in immersion oil. The content of fast green stained material in individual small lymphocytes was measured at 635 mμ with a microspectrophotometer (Olympus MSP-AIV). In each smear 30 cells were measured, and a mean value was calculated.

RESULTS

1. Lymphocyte blastogenesis after stimulation with PHA

Stimulation with PHA revealed a maximum response of blastogenesis at 72 hours of culture with percentage of the population response to PHA at 59.2±1.8 per cent (n=40). Smears obtained from the cultures at this stage showed clumps of cells containing large mononuclear forms

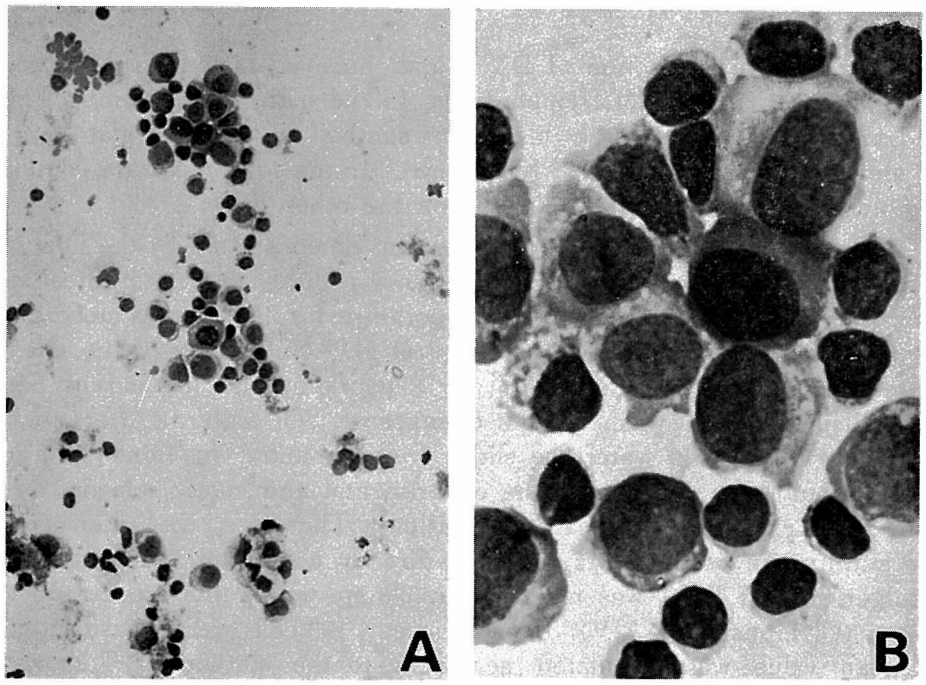

Fig. 1 Lymphoid cells in smear obtained from human peripheral blood lymphocytes cultured for 72 hours with PHA (10 μg/ml). Wright's stain. LC 79.
Fig. 1-A With PHA alone. ×200.
Fig. 1-B Higher magnification of Fig. 1-A. Note typical blastoid cells induced by PHA. ×1000.

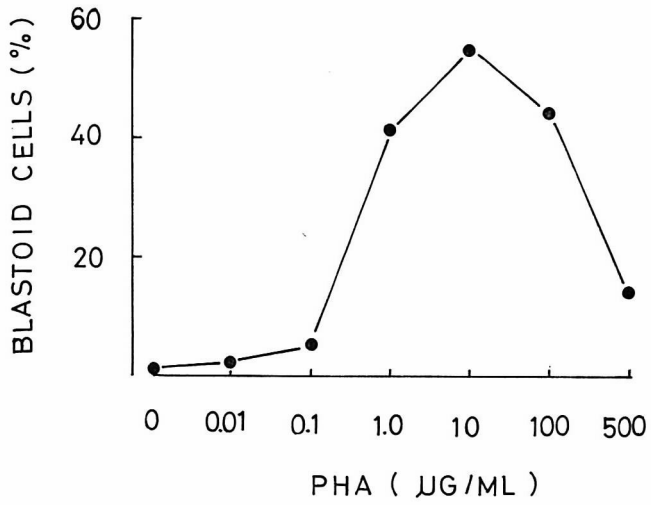

Fig. 2 Dose-response curve to PHA at 72 hours of human lymphocyte culture.

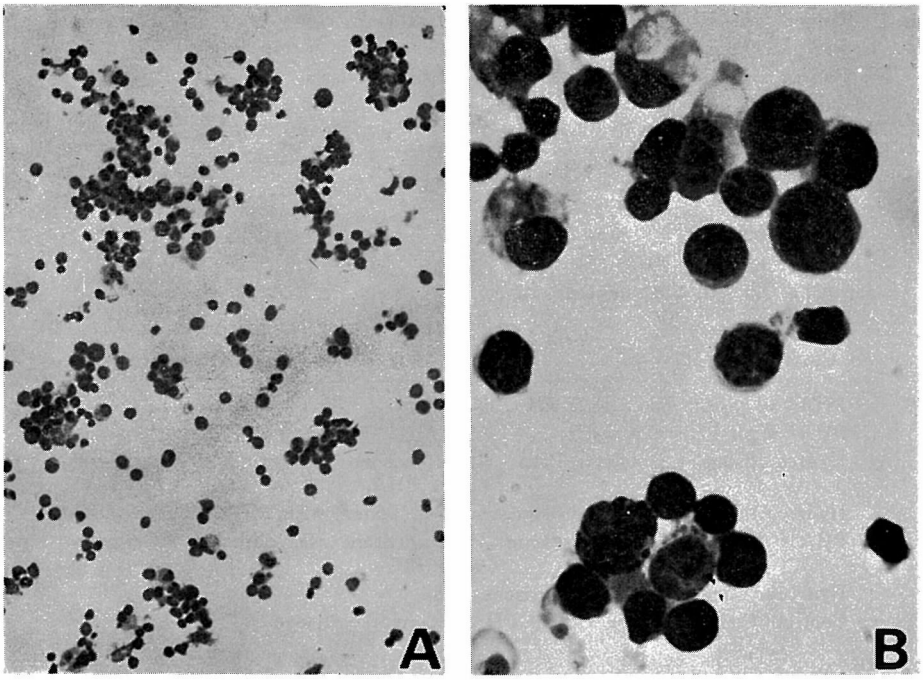

Fig. 3 Lymphoid cells in smear obtained from human peripheral blood lymphocytes cultured for 72 hours with PHA (10 μg/ml) and hydrocortisone (100 μg/ml). Wright's stain. LC 79.
Fig. 3-A With PHA and hydrocortisone simultaneously. ×200.
Fig. 3-B Higher magnification of Fig. 3-A. Note the marked inhibition of blastoid transformation by hydrocortisone. ×1000.

(mainly blastoid cells) interspersed with small lymphocytes (Fig. 1-A). The blastoid cells had a uniformly round nucleus with finely dispersed chromatin, one or more prominent nucleoli, and frequently contained a clear perinuclear zone (Fig. 1-B). The cytoplasm of these cells was intensely basophilic and pyroninophilic. Typically, blastoid cells from the PHA-stimulated cultures were 11-30 μ in diameter. The dose-response curve to PHA at 72 hours is shown in Fig. 2. The peak response was obtained at a concentration of 10 μg/ml.

2. Hydrocortisone inhibition of blastogenesis

The blastoid cells were rarely observed in the smear when lymphocytes were cultured with PHA and hydrocortisone simultaneously at 37°C for 72 hours (Figs. 3-A, B). However, the cultured lymphocytes appeared to still be viable (i.e. dye-excluding). In fact, I could not discern any significant difference between viability of lymphoid cells cultured

Table 1. Percentage of blastoid cells in PHA culture at 72 hours when hydrocortisone was added with PHA simultaneously or 30 minutes before or after PHA. All figures are mean values of percentage of blastoid cells. Mean±standard error (n=4)

Groups	Blastoid cells (%)
PHA	72.2 ± 5.3
Hydrocortisone — 30 min. — PHA	2.2 ± 0.4
Hydrocortisone+PHA	3.9 ± 2.2
PHA—30 min. — Hydrocortisone	7.0 ± 4.3
Hydrocortisone	0.5 ± 0.2
Control	1.0 ± 0.2

PHA : 10 μg/ml, Hydrocortisone : 100 μg/ml.
PHA : With PHA alone.
Hydrocortisone — 30 min. — PHA : Hydrocortisone was added at 30 minutes before PHA.
Hydrocortisone+PHA : Hydrocortisone was added with PHA simultaneously.
PHA — 30 min. — Hydrocortisone : Hydrocortisone was added at 30 minutes after PHA.
Hydrocortisone : With hydrocortisone alone.
Control : Control culture without PHA and hydrocortisone.

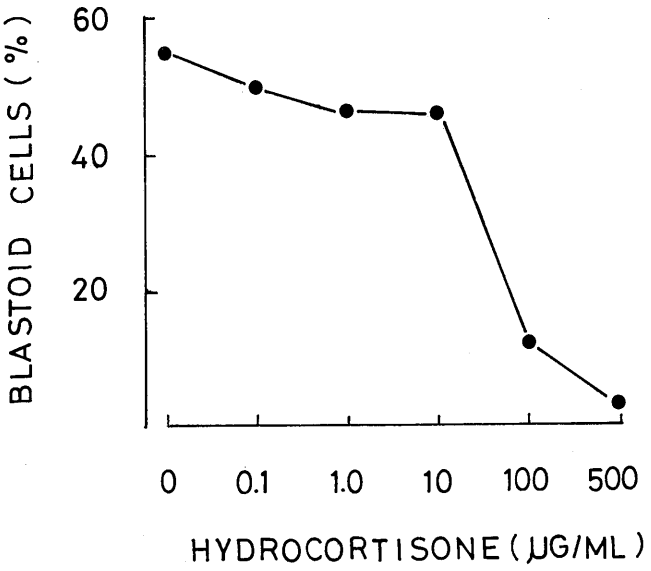

Fig. 4 Dose-response curve to hydrocortisone at 72 hours of human lymphocyte culture with PHA. Various concentrations of hydrocortisone were added with PHA (10 μg/ml) simultaneously.

with PHA and/or hydrocortisone for 72 hours. The hydrocortisone did not cause lysis or death of the lymphocytes over the range of 0.1-500 μg/ml. It was noted that hydrocortisone, in a concentration of 100 μg/ml, produced significant inhibition of lymphocyte blastogenesis by PHA when added not only simultaneously but also 30 minutes after PHA (Table 1). This concentration, therefore, seemed to be the most appropriate for observing the inhibitory effect of this steroid.

3. Effect of concentration of hydrocortisone on blastogenesis

The effect of various concentrations of hydrocortisone in PHA culture on lymphocyte blastogenesis was investigated (Fig. 4). The degree of inhibition of blastogenesis was not so large at concentration of hydrocortisone less than 10 μg/ml. Very high, supraphysiological concentrations of 100 to 500 μg/ml were found to be very inhibitory, however.

4. Effect of delayed addition of hydrocortisone on blastogenesis

To investigate the effect of delayed addition of hydrocortisone on lymphocyte blastogenesis hydrocortisone was administrated at various time intervals after PHA stimulation (Table 2). The later the addition of hydrocortisone, the less the inhibition of PHA-induced blastogenesis that occurred. When hydrocortisone was added within 1 hour after PHA stimulation blastogenic response to PHA was completely inhibited. A considerable degree of inhibition of blastogenesis was still observed when hydrocortisone was introduced 36 hours after PHA, but the inhib-

Table 2. Percentage of blastoid cells in PHA culture at 72 hours when hydrocortisone was added at various time intervals after PHA.

Time after PHA*	Blastoid cells (%)
0[1]	1.2
30 minutes	2.7
1 hour	1.3
3 hours	7.2
6 〃	10.1
12 〃	34.4
24 〃	40.2
36 〃	39.1
48 〃	63.3
60 〃	64.7
Control[2]	71.5

*Time of hydrocortisone administration after PHA.
[1]Hydrocortisone was administrated at the same time of PHA.
[2]PHA culture without hydrocortisone for 72 hours.

itory effect of the steroid was completely absent when added 48 hours after PHA stimulation.

5. Effect of removal of hydrocortisone from culture medium

To test the durability of the hydrocortisone effect on lymphocyte blastogenesis the following experiment was performed. An aliquot of lymphocyte suspension was allowed to incubate with hydrocortisone (100 μg/ml) for 30 minutes. Then the cells were centrifuged and resuspended three times in 5 ml of the Hanks solution for 5 minutes each. The cells were finally resuspended in 1 ml of the fresh culture medium and incubated with PHA (10 μg/ml) for 72 hours. As controls, PHA cultures with or without hydrocortisone were prepared. The degree of lymphocyte blastogenesis in these cultures was evaluated using the cells of 4 different persons (Table 3). The data obtained showed that the inhibitory effect of hydrocortisone practically disappeared by removing the hydrocortisone from the culture medium before stimulation with PHA.

Table 3. Effect on lymphocyte blastogenesis of removal of hydrocortisone from culture medium before PHA stimulation. Lymphocytes were incubated with hydrocortisone (100 μg/ml) at 37°C for 30 minutes. Then the hydrocortisone was removed from culture medium by washing and the lymphocytes were cultured PHA (10 μg/ml) for 72 hours (see text).

Specimen No.	Blastoid cells (%)			Recovery (%)
	No Hyc.	Hyc.	Hyc. removed	
1	56.9	1.1	32.5	57.1
2	78.4	2.1	69.6	88.8
3	73.0	2.5	65.4	89.6
4	63.9	2.0	47.3	74.0
Mean (4)	68.1±4.8	1.9±0.3	53.7±8.6	77.4±7.7

No Hyc : PHA culture without hydrocortisone.
Hyc : PHA culture with hydrocortisone.
Hyc removed : Hydrocortisone was removed from culture medium before PHA stimulation.
Recovery : Recovery (%) $= \frac{\text{Hyc removed}}{\text{No Hyc}} \times 100$

6. Effect of hydrocortisone on Feulgen-DNA content in PHA-stimulated lymphocytes.

The peripheral blood lymphocytes from one healthy person were examined for Feulgen-DNA content after culturing. The cultures were divided into 4 groups as shown in Fig. 5. Small lymphocytes from the

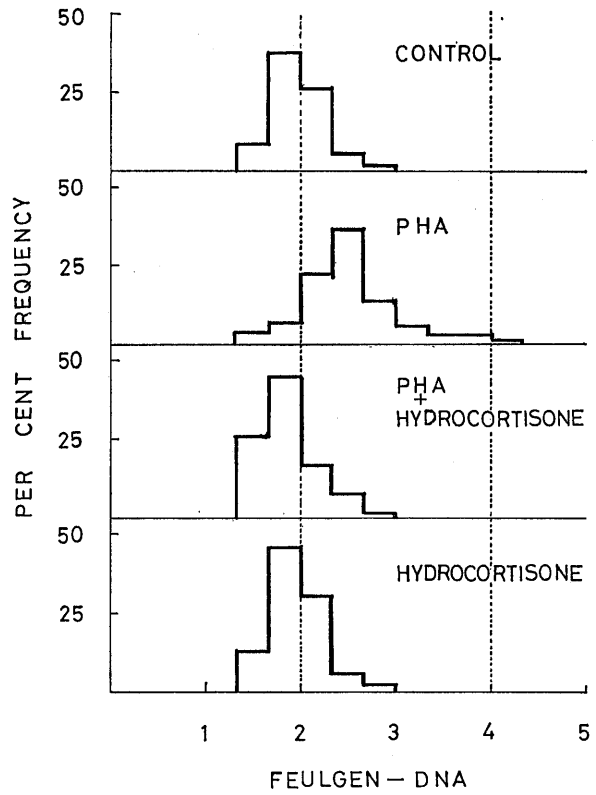

Fig. 5 Histogram of relative amount of Feulgen-DNA in lymphoid cells cultured for 72 hours after addition of PHA (10 μg/ml) and/or hydrocortisone (100 μg/ml).
Control : Control culture without PHA and hydrocortisone.
PHA : With PHA alone.
PHA+hydrocortisone : With PHA and hydrocortisone.
Hydrocortisone : With hydrocortisone alone.

control culture without PHA were seen to have a Feulgen-DNA content of diploid level (2c). However, in PHA-stimulated lymphocytes the range of Feulgen-DNA values was much wider, extending from a diploid level (2c) to tetraploid level (4c). This indicates that DNA synthesis proceeds in parallel with lymphocyte transformation after PHA stimulation. Such increases of DNA values were suppressed by the addition of hydrocortisone to PHA culture medium (Fig. 5). In addition, the Feulgen-DNA content of the lymphocytes in culture with only hydrocortisone was about the same as that of lymphocytes in control culture without PHA.

These findings show that hydrocortisone inhibits the DNA synthesis of PHA-stimulated lymphocytes and that in the concentration used here

it neither causes an increase nor decrease in the DNA content of small lymphocytes in control culture without PHA.

7. Effect of hydrocortisone of fast green histone content in PHA-stimulated lymphocytes

The lymphocytes from 1 person were examined for fast green histone content at various time intervals after incubation with PHA. A significant decrease in the relative amount of fast green stainable histone of small lymphocytes occurred 30 minutes after PHA stimulation. Three hours after PHA the histone content returned to the control value (Table 4).

Table 4. Relative amounts of fast green histone in small lymphocytes cultured with PHA (10 μg/ml). All figures are expressed as percent changes of the mean value in lymphocytes cultured without PHA. Mean±standard error (n=30 cells).

Time after PHA	% of control
Control*	100
15 minutes	92.4±3.9
30 minutes	75.2±2.5
1 hour	91.5±3.4
3 hours	98.3±4.3

*Control culture without PHA

Table 5. Effect of hydrocortisone on fast green histone content in small lymphocytes. All figures are mean values at 30 minutes after PHA administration and are expressed as percent changes of the mean value in lymphocytes cultured without PHA and hydrocortisone for same period. Mean±standard error (n=30 cells).

Groups	% of control
Control	100
PHA	82.0±7.2
Hydrocortisone — 30 min. — PHA	94.6±5.2
Hydrocortisone+PHA	104.6±3.4
Hydrocortisone	102.6±6.5

PHA : 10 μg/ml, Hydrocortisone : 100μg/ml.
Control : Without PHA and hydrocortisone.
PHA : With PHA alone.
Hydrocortisone — 30 min. — PHA : Hydrocortisone was added at 30 minutes before PHA.
Hydrocortisone+PHA : Hydrocortisone was added with PHA simultaneously.
Hydrocortisone : With hydrocortisone alone.

To test the effect of hydrocortisone on these histone changes after PHA, a related study was performed using lymphocytes from another person. The results are shown in Table 5. A similar decrease of fast green histone content of small lymphocytes was observed at 30 minutes after PHA stimulation. However, such a decrease in histone content was not seen when hydrocortisone was added with PHA simultaneously or 30 minutes before PHA. These findings suggest that the histone changes of lymphocytes in early stage after PHA may be closely related to DNA synthesis and that hydrocortisone inhibits these early events.

DISCUSSION

The present results confirm that hydrocortisone inhibits morphological transformation of lymphocytes in PHA culture (Figs. 3-A, B). That this inhibition was not caused by lysis or death of the lymphocytes was shown by the fact that the inhibitory effect of hydrocortisone practically disappeared when the hydrocortisone was removed from the culture medium before PHA stimulation (Table 3). This is in agreement with Shibata[17] and May et al.[18] These results suggest that the corticosteroid is only loosely bound to the binding sites of lymphocytes.

The extent of the inhibition of lymphocyte transformation depends on the concentration of hydrocortisone (Fig. 4). The inhibitory effect also depends on the time at which hydrocortisone is added after PHA stimulation—the later the addition of hydrocortisone the less inhibition was observed (Table 2). The effects of delayed addition of hydrocortisone in PHA culture medium on the blastogenesis were already reported[2-5], but the results among these authors are not necessarily in agreement.

A distinct but transient decrease in the relative amount of fast green histone of human small lymphocytes was observed at 30 minutes after PHA stimulation (Table 4). Such a decrease in histone content of kangaroo lymphocytes in PHA culture was also reported by Burton[19]. As it has been suggested that histone combines with DNA and suppresses DNA template activity[20], the above changes of histone content might reflect an early event in gene activation for DNA synthesis of lymphocytes after PHA stimulation. PHA may inhibit the suppressive effect of histone on DNA synthesis and thus induce the blastogenesis of lymphocytes. There are several reports on the inhibitory effects of corticosteroids on DNA synthesis[4,6,8,10,21], RNA synthesis[6-9], glucose metabolism[18] and stabilization of lysosomal membrane[22]. In this experiment, the histone changes of small lymphocytes in the early stages after PHA stimulation and subsequent increases in DNA synthesis of the lymphocytes were not

observed when hydrocortisone was added with PHA simultaneously or 30 minutes before PHA (Table 5, Fig. 5). The present study suggests that hydrocortisone inhibits the early events after PHA which are closely related to DNA synthesis.

SUMMARY

The effect of hydrocortisone on the blastogenesis of human peripheral blood lymphocytes in PHA culture was studied by morphological and cytochemical techniques.

Hydrocortisone markedly inhibited morphological transformation of lymphocytes in PHA culture for up to 72 hours. The extent of the inhibition of blastogenesis depends on the concentrations of the hydrocortisone and on the added time intervals after PHA stimulation. The inhibitory effect of hydrocortisone practically disappeared by removing the hydrocortisone from the culture medium before PHA stimulation.

A distinct, but transient, decrease in fast green histone content in small lymphocytes, about 75% of the control value, was obtained at 30 minutes after PHA stimulation. The increase in Feulgen-DNA content of the lymphocytes was clearly observed in PHA culture for 72 hours. However, such histone changes and thereafter increase of DNA value were not observed when hydrocortisone was added with PHA simultaneously or 30 minutes before PHA. Present results suggest that hydrocortisone may inhibit histone changes of small lymphocytes in early stages after PHA, and suppresses DNA synthesis of the PHA-stimulated lymphocytes.

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