Studies on Inhibition of Human Lymphocyte Blastogenesis by Hydrocortisone in vitro

II. Effect of Hydrocortisone on the Mixed Lymphocyte Reaction

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The preparation of a mixed lymphocyte culture (MLC) from the cells of two different individuals usually results in a blastogenic response with cell proliferation (Bain et al. 1964[1], Bach and Hirschhorn 1964[2]). This in vitro mixed lymphocyte reaction (MLR) reflects the individual's capacity to identify foreign cells and serves as a model of the recognition phase of the homograft reaction.

In a previous report (Kato, 1977[3]) the author has shown that the blastogenesis of human lymphocytes in PHA cultures is inhibited by the addition of hydrocortisone to the culture medium; the author hypothesized that the inhibition of blastogenesis may be due to the suppression of early events in the lymphocytes, such as changes in membrane and changes in the binding of histone to DNA, which usually occur soon after the addition of PHA to lymphocyte cultures and which are closely related to DNA synthesis. Some authors have reported that the MLR was also inhibited by glucocorticoids (Vas and Lowensterin 1965[4], Heilman and Leichner 1972[5]). These findings suggest that hydrocortisone may block the capacity of responding lymphocytes to identify foreign cells and to transform themselves into large blastoid cells by a mechanism similar to that in the inhibition of PHA response. The present study is an attempt to explore this possibility. To this end, the effects of hydrocortisone on the MLR, both concentration of dose and the time of its administration, were examined.

MATERIALS AND METHODS

Test materials: Hydrocortisone (Fluka AG, Buchs SG, Switzerland) was prepared according to the same method previously described[3]. The steroid was dissolved in 95% ethanol and diluted with sterile distilled water to give a final concentration of 10 μg/ml. When necessary, further

dilutions were made with Hanks balanced salt solution (Difco).

Lymphocyte separation: Fifteen milliliters of human blood from healthy adult male volunteers were drawn aseptically into a syringe containing 300 units heparin (heparin natrium, Takeda). Lymphocytes were separated from the heparinized blood by a gradient separation method using Ficoll-Isopaque solution (Lymphoprep, Nyeggard & Co. As., Oslo) as previously described[3]. The lymphocytes obtained were washed with chilled Hanks balanced salt solution and finally resuspended at a concentration of 2×10^6 cells/ml in TC-199 medium (Chiba) with 20% calf serum containing 100 units of penicillin (Meiji) and 100 μg/ml of streptomycin (Meiji).

Lymphocyte culture: Each-half milliliter of lymphocyte suspension from the two donors was added to a 14×100 mm sterile test tube with Morton stainless steel cap. The cells were cultured for 7 days at 37°C in a 95% air-5% CO_2 humid atmosphere. The hydrocortisone to be used, unless otherwise noted, was added to 1 ml of cell suspension in a volume of 0.01-0.05 ml. Control cultures, containing lymphocytes of each donor separately, were set up and continued in the same manner.

Harvesting of cultures: When the cultures were terminated at the 7th day, the cells from each test tube were washed twice with 5 ml of Hanks solution by centrifugation at 700 rpm for 10 minutes and then resuspended in 1 ml of Haemaccel (Hoechst). After centrifugation of the Haemaccel cell suspensions at 1000 rpm for 10 minutes the cell pellets were smeared onto a slide for estimation of the rate of blastogenesis. The percentage of blastoid cells was evaluated as the index of blastogenesis according to the method previously described[3].

Quantitative DNA estimation: The DNA of the cultured lymphocytes was demonstrated by the Feulgen reaction (Feulgen and Rossenbeck 1924[7], Lessler 1953[8]) after hydrolysis with 5 N HCl as described by Tomonaga et al.[9,10]. The intensity of stainability for Feulgen reaction was measured at a wave length of 560 mμ on groups of 100 cells each by microspectrophotometer (Olympus MSP-AIV). The relative amount of Feulgen-DNA was calculated by multiplying the rate of extinction by the nuclear area according to the method of Tomonaga et al.[11] and expressed in arbitrary units.

RESULTS

1. Blastogenesis in MLC (Figs. 1 and 2)

Morphologically, the blastoid cells in MLC resembled those observed

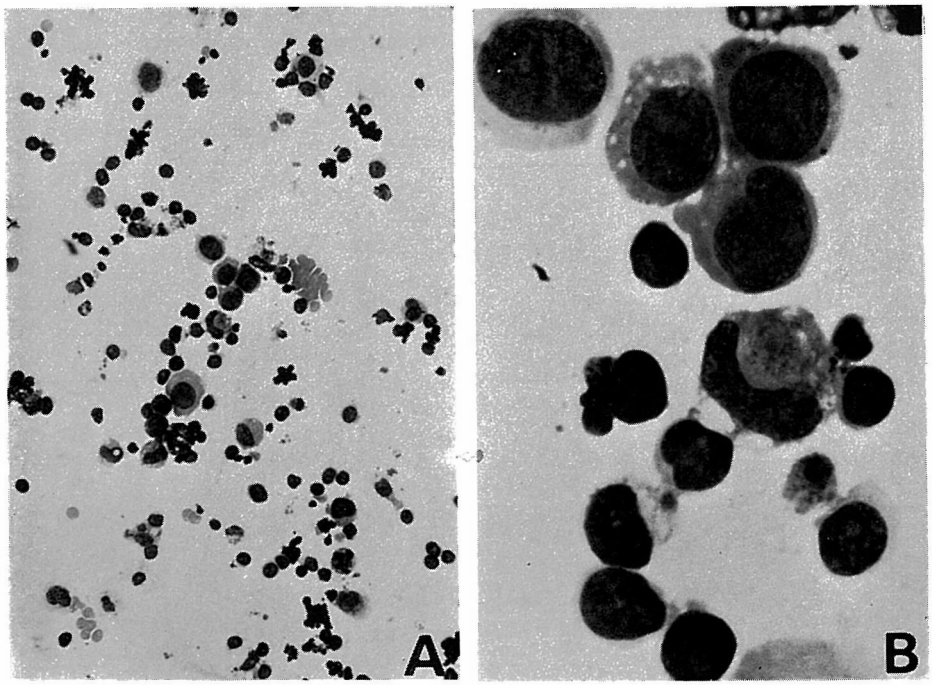

Fig. 1. Lymphoid cells in smear obtained from mixed human lymphocyte culture after 7 days incubation without hydrocortisone. Wright's stain, MLC 28.
Fig. 1-A Without hydrocortisone. ×200.
Fig. 1-B Higher magnification of Fig. 1-A. Note typical blastoid cells as a result of the MLR. ×1000.

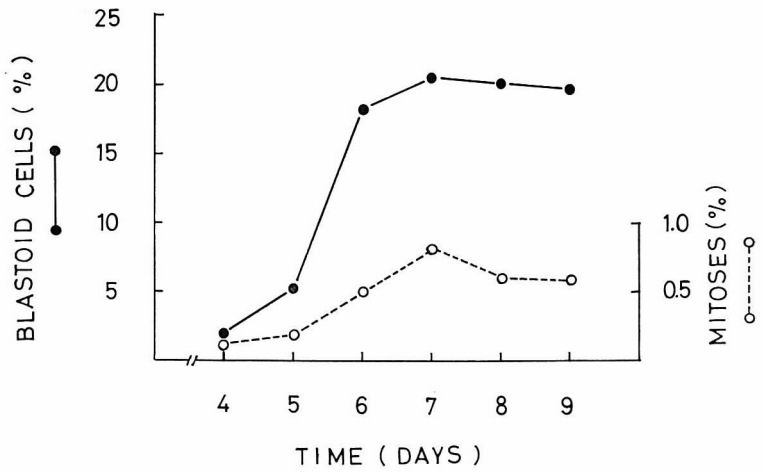

Fig. 2. Percentage of blastoid cells and mitoses in mixed culture from 4 to 9 days after the onset of the culture.

Table 1 Percentage of blastoid cells on the 7th day after the onset of mixed lymphocyte culture. Hydrocortisone was added at the onset of the culture.

MLC No.	Mixed culture	Mixed culture +Hydrocortisone	Unmixed control
1	10.2	0.9	0.4
2	22.4	2.5	0.5
3	19.2	4.7	1.5
4	14.5	1.1	0.5
5	20.7	1.7	0.6
Mean	17.4±2.2	2.2±0.7	0.7±0.2

Hydrocortisone: 100μg/ml.

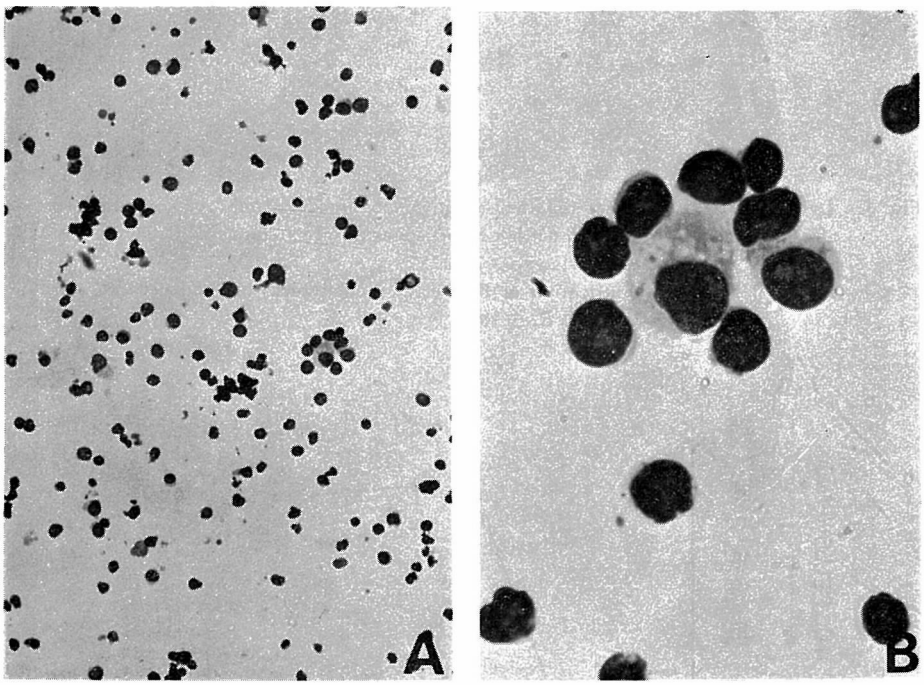

Fig. 3. Lymphoid cells in smear obtained from mixed human lymphocytes culture after 7 days incubation with hydrocortisone.
Fig. 3-A With hydrocortisone (100 μg/ml). ×200.
Fig. 3-B Higher magnification of Fig. 3-A. Note no blastoid cells. ×1000.

in PHA culture. However, the rate of blastoid transformation in MLC was consistently smaller than that noted after stimulation with PHA and the transformation reached a peak at 7 days while PHA culture revealed a maximum at 3 days. The MLR from two different donors was much greater than that in the unmixed control culture.

2. Effect of concentration of hydrocortisone on MLR

The effect of the addition of 100 μg/ml of hydrocortisone at the onset of culture was examined in the 5 MLCs. The details of the experiments are shown in Table 1. The results indicate that the MLR is markedly inhibited by hydrocortisone, though the rate of blastogenesis was greater than that obtained in the unmixed control culture. Fig. 3 shows the suppression of blastoid transformation in a MLC due to addition of hydrocortisone. Another experiment was performed on two MLCs to test the effect of various concentrations of hydrocortisone on the MLR (Table 2). The smallest concentration of hydrocortisone which caused an obvious decrease in the number of blastoid cells formed was 0.1 μg/ml; the inhibitory effect of the lowest concentration (0.01 μg/ml) of the steroid was unnoticeable.

3. Effect of delayed addition of hydrocortisone on MLR

To investigate the effect of delayed addition of hydrocortisone (100 μg/ml) on the MLR, hydrocortisone was added at various time intervals after the onset of the culture. The results obtained are shown in Table 3 and Fig. 4. If the hormone was added within 24 hours after the start of culture the MLR was markedly suppressed. However, there was less

Table 2 Percentage of blastoid cells in mixed lymphocyte culture on the 7th day after the onset of the culture with various concentrations of hydrocortisone. The steroid was added at the onset of the culture.

Hydrocortisone (μg/ml)	Blastoid cells (%)	
	MLC 1	MLC 2
0.01	18.4	20.5
0.1	12.5	16.4
1.0	10.3	10.5
10	9.0	6.2
100	4.7	1.7
500	1.4	0.3
Control*	19.2	20.7

*Mixed lymphocyte culture without hydrocortisone.

Table 3 Percentage of blastoid cells in mixed lymphocyte culture on the 7th day after the onset of the culture. Hydrocortisone was added at various time intervals after the onset of the culture.

Time of hydrocortisone addition after onset of mixed culture	Blastoid cells (%)
0[1)]	1.9
1 day	5.8
2 days	10.6
3 〃	9.8
4 〃	15.5
5 〃	16.8
6 〃	17.9
Control	18.4

Hydrocortisone: 100 μg/ml.

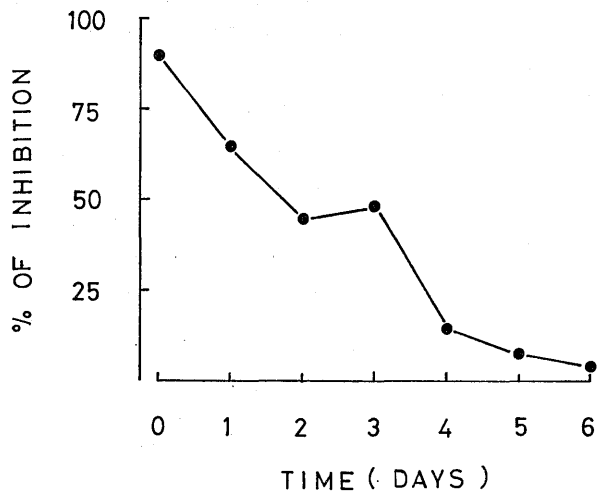

Fig. 4. Effect of delayed addition of hydrocortisone on the MLR. Hydrocortisone (100 μg/ml) was added at various time intervals after the onset of the culture. Estimation of blastogenesis was made at 7th day after the onset of MLC.

inhibition of the MLR when the addition of the steroid was delayed 2 days after the onset of the culture. Waiting for 4 days after the onset of culture before adding hydrocortisone resulted in a substantial loss of the inhibitory effect. These data show that the time of addition of hydrocortisone may be important for the steroid inhibition of the MLR.

4. Effect of hydrocortisone on Feulgen-DNA content of lymphocytes in MLR

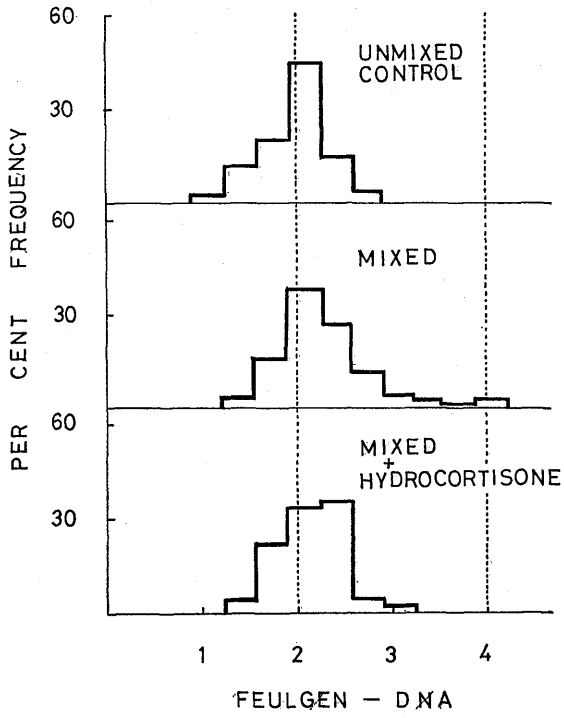

Fig. 5. Histogram of the relative amount of Feulgen-DNA in the lymphoid cells in MLC with or without hydrocortisone (100 μg/ml) after 7 days.
Unmixed control: Unmixed control culture without hydrocortisone.
Mixed: Mixed culture without hydrocortisone.
Mixed+Hydrocortisone: Mixed culture with hydrocortisone.

The relative amounts of Feulgen-DNA of lymphoid cells in the smear of 7 day old MLC incubated both with or without hydrocortisone (100 μg/ml) are shown in Fig. 5. A marked decrease in the Feulgen-DNA content of the cells was observed in the MLC with hydrocortisone. The percentage of the cell population which had tetraploid level (4c) was significantly reduced in the MLC with hydrocortisone as compared with the MLC without hydrocortisone. These results indicate that hydrocortisone may inhibit DNA synthesis of lymphocytes stimulated by allogeneic cells.

5. Effect of removal of hydrocortisone from culture medium on MLR

Mixed lymphocyte suspensions from two different individuals were allowed to incubate with 100 μg/ml of hydrocortisone. After 30 minutes the cells were washed three times with 5 ml of Hanks solution to remove the hydrocortisone from the culture medium. Then, the MLC was continued for 7 days. The experiments were performed on the pairs. The

Table 4 Effect of the removal of hydrocortisone from mixed lymphocyte culture medium on lymphocyte blastogenesis. 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 for 7 days (see text).

MLC No.	Blastoid cells (%)			Recovery (%)
	No Hyc	Hyc	Hyc removed	
1	15.5	2.1	13.5	87.1
2	11.7	1.5	10.1	86.3

No Hyc: Mixed culture without hydrocortisone.
Hyc: Mixed culture with hydrocortisone.
Hyc removed: Hydrocortisone was removed from culture medium.

$$\text{Recovery } (\%) = \frac{\text{Hyc removed}}{\text{No Hyc}} \times 100$$

results obtained are shown in Table 4. The data show that inhibitory effect of hydrocortisone on the MLR was almost entirely eliminated by procedure.

DISCUSSION

The marked inhibition of hydrocortisone upon lymphocyte transformation in MLC was observed in the present study (Table 1 and Fig. 3). The inhibitory effect of hydrocortisone on blastogenesis was found to extend even to the lower concentrations of 0.1-1.0 μg/ml. These concentrations of hydrocortisone were significantly lower than those necessary to inhibit blastogenesis of PHA-stimulated lymphocytes as reported by Schiff et al.[6].

The inhibitory effect of hydrocortisone on blastogenesis in MLC was progressively lost with the increasing lapse of time between the onset of the culture and the time at which hydrocortisone was added (Table 3 and Fig. 4).

The relative amount of Feulgen-DNA of the lymphocytes in mixed culture with hydrocortisone was observed to be significantly lower than that of the cells in MLC without hydrocortisone (Fig. 5). Such a decrease in the Feulgen-DNA content of the lymphocytes in MLC with hydrocortisone reflects the decrease of DNA synthesis in the lymphocytes. These results suggest that hydrocortisone inhibits DNA synthesis of the lymphocytes in mixed culture. These inhibitory effects have been corroborated by using the method of measuring ^{3}H-thymidine incorpora-

tion for DNA synthesis[4,5].

Hydrocortisone may be only loosely bound to the binding sites of the lymphocytes since it was easily removed from the culture medium. Thus, prolonged incubation with hydrocortisone seems to be necessary to suppress blastogenesis in MLC.

Recently it has been suggested that genes which map in the I region, and are expressed in the lymphocyte, are responsible for the stimulation of allogeneic responding cells. Studies on the hydrocortisone effect involving this point are now in progress in our laboratory.

SUMMARY

The effect of hydrocortisone on the mixed lymphocyte reaction (MLR) in human peripheral blood was studied by morphological and cytochemical techniques.

Hydrocortisone markedly inhibited the lymphocyte transformation in mixed lymphocyte culture (MLC).

The extent of the inhibition of blastogenesis depended on the concentration of hydrocortisone. Those concentrations of hydrocortisone that inhibited blastogenesis in MLC were significantly lower than those necessary to inhibit PHA induced blastogenesis.

The inhibitory effect of hydrocortisone also depended on the interval of time at which it was added after the onset of the MLC.

A substantial loss of the inhibitory action of hydrocortisone upon the MLR was seen when the steroid was removed by washing 30 minutes after the onset of the culture.

The increase in Feulgen-DNA content of the lymphocytes was clearly observed in MLC. Such an increase of the DNA value was not observed in 7 day old MLC with hydrocortisone. Especially a population of cells which contain tetraploid level (4c) was considerably reduced.

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