Biochemical Pharmacology, Vol. 17, pp. 227–234. Pergamon Press. 1968. Printed in Great Britain

INHIBITORY ACTION OF HYDROXYUREA ON NUCLEIC ACID SYNTHESIS IN CULTURES OF HUMAN LYMPHOCYTES STIMULATED BY POKEWEED MITOGEN

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(*Received* 29 *June* 1967; *accepted* 11 *October* 1967)

Abstract—Measurement of the uptake of [^{3}H]-thymidine and [^{3}H]-uridine was used to investigate the effects of hydroxyurea on nucleic acid synthesis in cultures of human lymphocytes stimulated by pokeweed mitogen. Hydroxyurea caused reversible inhibition of DNA synthesis, and inhibition of both the RNA synthesis occurring during DNA synthesis, and that taking place before the onset of DNA synthesis in these cultures.

HYDROXYUREA has been shown to inhibit the growth of various biological systems,[1, 4] is used in the chemotherapy of certain forms of human cancer[5] and has been shown to have some immunosuppressive activity.[6] Several workers have demonstrated that hydroxyurea causes a rapid inhibition of DNA synthesis that is readily reversible by washing.[3, 7, 8] A large inhibition of DNA synthesis is caused by doses of hydroxyurea that have little or no effect on RNA synthesis or protein synthesis[3, 8] and it has been suggested[9] that the slight inhibition of RNA synthesis observed in some systems may result from the inhibition of DNA synthesis. Pfeiffer and Tolmach,[10] using synchronised cultures of HeLa cells, showed that a dose of hydroxyurea causing almost complete inhibition of DNA synthesis, caused little or no inhibition of RNA synthesis in cells in the pre-DNA-synthetic period of the cell cycle (G_1), although it did inhibit an increase in RNA synthesis beginning early in the S phase of the cell cycle.

Human small lymphocytes grow and divide *in vitro* in response to various types of stimuli.[11, 12] This phenomenon is sometimes referred to as lymphocyte transformation. Cultures of stimulated lymphocytes have a time sequence of growth that is of particular value in the study of certain types of drug action. After the addition of the stimulus the responding lymphocytes show increased RNA synthesis for several hours before DNA synthesis begins. As a consequence of this it is possible to make use of the system for investigation of the effect of drugs on RNA synthesis that is not linked with DNA synthesis.

Biochemical changes occur in the lymphocytes very rapidly after the addition of a stimulating agent[13] and it has been shown that a short incubation with phytohaemagglutinin (PHA)[14] or pokeweed mitogen (PWM)[15] is sufficient to initiate subsequent growth and division in a large proportion of the lymphocytes. This characteristic enables the stimulated lymphocyte system to be used for investigation of the effects

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of drugs on the initiation of stimulation, and may be of relevance to the study of immunosuppression.

In the present experiments PWM-stimulated cultures of human lymphocytes were used for an investigation of the effects of hydroxyurea on DNA synthesis, and on RNA synthesis during DNA synthesis and before DNA synthesis had begun. Some observations were made on the effect of hydroxyurea on lymphocyte transformation and on the cytotoxicity of the drug. PWM was chosen as the stimulating agent in preference to PHA because, as has been shown previously,[15] the lesser degree of leucoagglutination caused by PWM enables better quantitation of the lymphocyte response.

METHODS

Preparation of cultures

Cell suspensions in which lymphocytes represented more than 99 per cent of the total leucocytes, were obtained from 50 to 70 ml of venous blood.[16] Except in experiments in which the cells were to be washed, the lymphocyte concentration of the supernatant obtained after sedimentation was adjusted to $1{\cdot}5 \times 10^6$/ml with TC 199, and PWM was added to the culture.

In experiments in which the cells were to be washed, the initial lymphocyte suspension was centrifuged for 10 min at 135 *g* and the suspending medium was removed and diluted with TC 199. Half the volume was used to re-suspend the cells to an appropriate lymphocyte concentration, and PWM was added to the culture. The remaining half of the diluted suspending medium was retained for re-suspension of the cells after the washing procedure. In experiments in which cells were washed immediately after a 30 min incubation with PWM, the initial lymphocyte concentration was adjusted to $1{\cdot}8 \times 10^6$/ml, so that after cell loss caused by the washing, the cultures would contain approximately $1{\cdot}5 \times 10^6$ lymphocytes/ml.

PWM (The Grand Island Biological Co., Grand Island, New York) was re-constituted with sterile saline. The dose used was that recommended by the manufacturers.

All cultures were grown at 37°. The cultures (25–50 ml) were contained in siliconised screw-capped glass bottles (4 oz) until immediately before the addition of hydroxyurea when they were divided as 3 ml aliquots in Pyrex screw-capped tubes, 16 × 125 mm.

Hydroxyurea (E. R. Squibb & Sons, New York, or Sigma Chemical Co., St. Louis, Missouri) was dissolved in sterile saline.

In order to wash hydroxyurea from a culture, the medium in which the cells were suspended was removed after centrifugation, and the cells in each tube were washed with 9 mls TC 199, centrifuged again and finally re-suspended in 3 ml of the original, cell-free suspending medium that had been set aside at the beginning of the experiment. Each centrifugation was for 7 min at 135 *g*.

Cell counts and morphological assessment of transformation

Cell counts and morphological assessment of transformation were made as described previously.[15]

Uptake of [^{3}H]*-uridine or* [^{3}H]*-thymidine*

[^{3}H]-uridine (nominally 5:6-^{3}H; 2·1 c/mM) and [^{3}H]-thymidine (2·9 c/mM) were obtained from the Radiochemical Centre, Amersham, Bucks. The cultures were

incubated at 37° with the isotope (2 μc/ml) for 1 hr, and the incorporation of radioactivity was measured by scintillation counting as described previously.[15]

Estimation of hydroxyurea

Samples of test and control mixtures were diluted 10-fold with 0·1 M phosphate buffer, pH 8·0, and the hydroxyurea contents were determined on as described previously.[17] Test mixtures consisted of a PWM stimulated culture of lymphocytes to which hydroxyurea $1{\cdot}2 \times 10^{-}$M had been added at the same time as the PWM. Control mixtures consisted of TC 199 containing PWM and hydroxyurea in the same concentrations as in the controls.

RESULTS

Stability of hydroxyurea in cultures of PWM-*stimulated lymphocytes*

Estimation of hydroxyurea in a culture to which hydroxyurea $1{\cdot}2\times 10^{-3}$M had been added at the same time as PWM, showed that the hydroxyurea concentration did not change during 3 days of culture.

Effect of hydroxyurea on cell number

When hydroxyurea $1{\cdot}2 \times 10^{-2}$M was added to 4 tubes of a lymphocyte culture at the same time as PWM, and the total nymbers of lymphocytes in these tubes at 24 and 48 hr were compared with those in 4 untreated tubes of PWM-stimulated lymphocytes from the same culture, no difference could be detected. Thus, hydroxyurea does not cause any marked cell destruction during the various incubation schedules used in these experiments.

Inhibition of [^3H]-*thymidine incorporation into DNA*

Inhibition of [^{3}]H-thymidine incorporation into the DNA of lymphocytes of a 48 hr stimulated lymphocyte culture was shown to result when hydroxyurea was added 4 hr before a 1-hr period of labelling with [^{3}H]-thymidine. The inhibition was approximately linearly related to the log dose of hydroxyurea. In the experiment shown (Fig. 1) a 50 per cent reduction was obtained with a dose of $1{\cdot}2 \times 10^{-4}$N. In another experiment the value obtained was $1{\cdot}1 \times 10^{-4}$M.

Recovery of DNA *synthesis after removal of hydroxyurea*

In a 48 hr culture, the effect on [^{3}H]-thymidine uptake of a 2 hr exposure to hydroxyurea, could be abolished by washing the cells free of hydroxyurea with TC 199 and re-suspending them in a portion of the original medium that had been set aside at the beginning of the culture, before the addition of stimulant: in cultures incubated for 1 hr after removal of the hydroxyurea, before a 1 hr period of labelling with [^{3}H]-thymidine, [^{3}H]-thymidine incorporation had returned to control levels.

On the other hand, washing the cells did not lead to a full restoration of [^{3}H]-thymidine incorporation to control levels within 1 hr when the cells had been incubated with hydroxyurea (6×10^{-4}M) from the beginning of the culture.

Transformation in the presence of hydroxyurea

The addition of hydroxyurea $1{\cdot}2 \times 10^{-3}$M to a culture at the same time as PWM, did not reduce the number of small lymphocytes transforming in response to the PWM

stimulation. At 48 hr, in these cultures, exposed to hydroxyurea continuously, there was still a little DNA synthesis (approximately 6 per cent of that in aliquots of the culture not exposed to hydroxyurea).

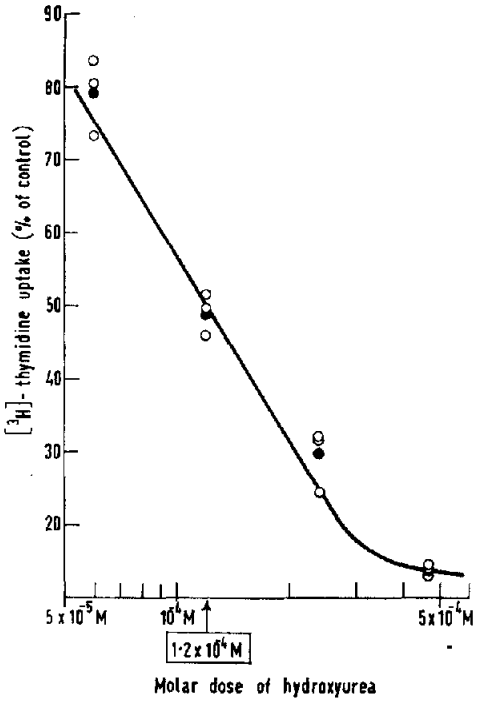

FIG. 1. Effect of a 4 hr exposure to hydroxyurea on [^{3}H]-thymidine uptake in a culture of lymphocytes stimulated with PWM for 48 hr. ○—result from an individual tube; ●—mean result. Control mean, cpm = 10,953.

Inhibition of incorporation of [^{3}H]*-uridine into* RNA

[^{3}H]-uridine uptake was not significantly reduced in a 48 hr PWM-stimulated culture, when hydroxyurea, $1{\cdot}2 \times 10^{-2}$M was added at the beginning of a 1 hr period of labelling with the isotope, but [^{3}H]-uridine was reduced to 35·5 per cent of the controls when the hydroxyurea was added 4 hr prior to the beginning of the labelling period, even though the uptake was still almost twice that that occurred (15·1 per cent of the controls) when the hydroxyurea was present from the beginning of the culture. (Each value = mean of 4 tubes. Mean control value = 9457 cpm).

The decrease in [^{3}H]-uridine incorporation caused by addition of hydroxyurea 4 hr before the beginning of a 1-hr labelling period in 48 hr PWM-stimulated culture was shown to be approximately linearly related to log dose between 6×10^{-4}M and $1{\cdot}2 \times 10^{-2}$M. A 50 per cent decrease was caused by a dose of $3{\cdot}2 \times 10^{-3}$M in this experiment, whose results are shown in Fig. 2. Another experiment gave a value of $5{\cdot}8 \times 10^{-3}$M.

Exposure to hydroxyurea beginning 4 hr before a 1-hr period of labelling with [^{3}H]-uridine in a 24 hr culture, also caused a reduction in [^{3}H]-uridine incorporation which was approximately linearly related to log dose of hydroxyurea. However, a dose of

$3{\cdot}2 \times 10^{-3}$M, which caused 50 per cent reduction in [^{3}H]-uridine uptake into a 48 hr culture (Fig. 2) caused a reduction of only approximately 20 per cent in a 24 hr culture (Fig. 3). A similar degree of sensitivity of hydroxyurea was shown in two other experiments.

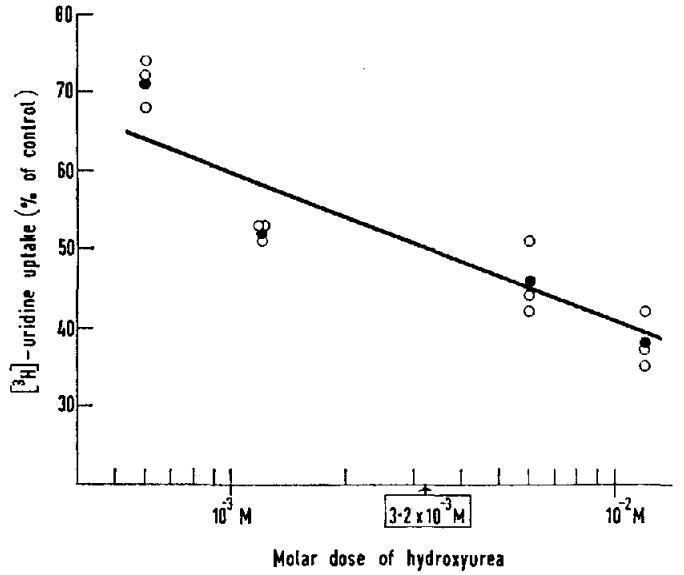

FIG. 2. Effect of a 4 hr exposure to hydroxyurea on [^{3}H]-uridine uptake in a culture of lymphocytes stimulated with PWM for 48 hr. ○—result from an individual tube; ●—mean result. Control mean cpm = 68,187.

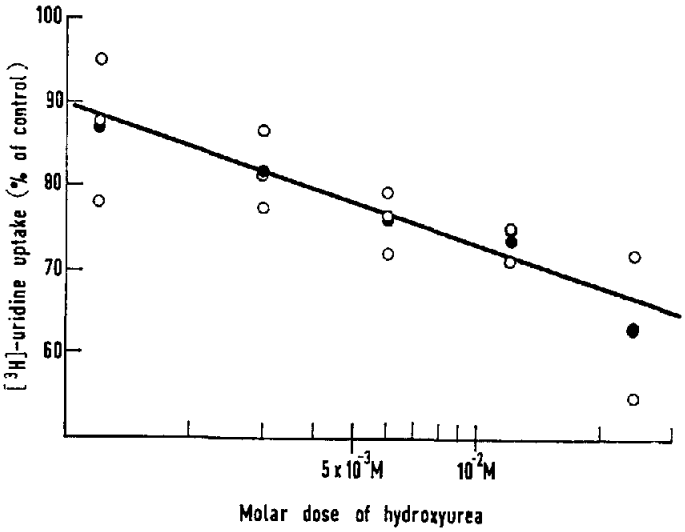

FIG. 3. Effect of a 4 hr exposure to hydroxyurea on [^{3}H]-urindine uptake in a culture of lymphocytes stimulated with PWM for 22 hr. ○—result from an individual tube; ●—mean result. Control mean cpm = 15,538.

Contribution of RNA *synthesis in small lymphocytes to total* RNA-*synthesis in a* PWM-*stimulated lymphocyte culture*

The proportion of small lymphocytes in a culture varies with time[15] but the contribution of small lymphocytes to the total RNA synthesis of a stimulated lymphocyte culture is very small. This was demonstrated by autoradiography: in an autoradiograph of a 48 hr culture labelled for 15 min with [^{3}H]-uridine, the mean grain count for transformed cells was 23 and that for small lymphocytes only 0·4.

Effect of the presence of hydroxyurea $1{\cdot}2 \times 10^{-2}$M *during stimulation and the growth phase preceding the onset of* DNA *synthesis*

When hydroxyurea $1{\cdot}2 \times 10^{-2}$ M, added to a culture at the same time as PWM, was removed 24 hr later and [^{3}H]-thymidine incorporation was measured at 48 hr, it was found that [^{3}H]-thymidine incorporation was reduced compared with controls (Mean value of controls = 17,575 cpm; mean value of treated* This was also true when hydroxyurea was present during a ½ hr stimulation with PWM and for 1 hr previously. It was not possible to demonstrate an effect on RNA synthesis at 2 days.

The presence of hydroxyurea during the initiation of stimulation in a culture of lymphocytes, and during the period of growth prior to DNA synthesis, did not appear to have an effect on subsequent DNA synthesis unless the dose of hydroxyurea was of a size to inhibit RNA synthesis.

DISCUSSION

Following the gene de-repression in small lymphocyte nuclei that is initiated by a mitogenic stimulus, there is a marked increase in growth of the cells, which involves RNA synthesis, protein synthesis and the formation of new organelles. In PWM-stimulated lymphocytes this period of growth lasts approximately 24 hr prior to the onset of DNA synthesis.[18] In the cultures examined approximately 25 hr after the addition of PWM, the incorporation of [^{3}H]-uridine into RNA took place in cells in the growth phase prior to the onset of DNA synthesis and, to a much lesser extent, into untransformed small lymphocytes. In the cultures examined at 48 hr [^{3}H]-uridine was also incorporated into cells in the DNA-synthetic period of the cell cycle (S) and the post DNA-synthetic period (G_2).

Hydroxyurea causes an inhibition of [^{3}H]-uridine incorporation into RNA in stimulated lymphocyte cultures at 48 hr when there are cells in all phases of the cell cycle, but the dose required to cause 50 per cent inhibition is more than 10 times that necessary to cause a comparable inhibition of [^{3}H]-thymidine incorporation into DNA.

Transformed lymphocytes, in common with a number of other cell systems, show a marked increase in RNA synthesis commencing at the beginning of the S period and continuing throughout G_2.[19] A similar observation was made[20] in a Chinese hamster cell line. Pfeiffer and Tolmach[10] found that in synchronised HeLa cells, hydroxyurea, and other inhibitors of DNA synthesis, caused inhibition of the increase of RNA synthesis beginning at the start of the S period in these cells. Their results appeared to indicate that hydroxyurea did not cause an inhibition of RNA synthesis independent of its effect on DNA synthesis. This is not so in the case of stimulated lymphocytes, since in a 24 hr culture, in which DNA synthesis is negligible, [^{3}H]-uridine uptake can be inhibited by hydroxyurea (Fig. 3), although higher doses are required than in a

* Samples = 5514 cpm = 31·4 per cent of controls.

48 hr culture. It has been suggested[21] that the newly synthesised DNA in cells in the S and G_2 periods, is active for RNA coding, and that this may account for the increased RNA synthesis in these cells compared with G_1 cells. However, this theory does not explain the difference in sensitivity to hydroxyurea, of RNA synthesis in the pre-DNA synthetic cells of a 24 hr culture and in the mixed population of a 48 hr culture.

In cultures of stimulated lymphocytes, as in other cell systems, hydroxyurea causes an inhibition of DNA synthesis. The dose of hydroxyurea causing 50 per cent inhibition of [^{3}H]-thymidine incorporation was of the same order as that described for other mammalian cell systems.[1, 9, 3] Also as shown by other workers using other experimental cell systems,[3, 7, 8] if DNA synthesis is inhibited by a short incubation with hydroxyurea it is possible to restore the uptake of [^{3}H]-thymidine to normal levels by removing the drug, but in our experiments, 1 hr after the removal of hydroxyurea from a culture exposed to hydroxyurea from the beginning of the culture, [^{3}H]-thymidine uptake was still partially depressed.

Sinclair[7] used plating techniques with cultures of Chinese hamster cells to show that a short incubation with hydroxyurea had a selective cytotoxic action on S cells, even though DNA synthesis returned to normal levels very shortly after removal of the drug. He suggested that hydroxyurea might be an effective synchronising agent for asynchronous cell populations. Cultures of stimulated lymphocytes survive for only a few days and are not suited to demonstration of drug effects on cell survival by plating techniques, but a cytotoxic action of hydroxyurea on stimulated lymphocytes has been demonstrated as chromosome damage.[22] However, since in our experiments no decline in cell number could be detected to result from the presence of hydroxyurea throughout a culture period of 3 days, hydroxyurea would not be expected to be an effective synchronising agent for cultures of stimulated lymphocytes.

It is difficult to interpret the cause of the inhibition of [^{3}H]-thymidine incorporation at 48 hr when hydroxyurea $1{\cdot}2 \times 10^{-2}$ M had been present during the first 24 hr of culture, since washing of the cells had to be kept to the minimum to minimise cell loss and consequent tube-to-tube variation, and it is probable that at least a part of the effect obtained was due to the amount of hydroxyurea left after washing of the cells, being sufficient to inhibit DNA synthesis directly.

It has not been possible to show an effect of the presence of hydroxyurea during the stimulation period alone on subsequent RNA synthesis. However, a slight effect may have been masked by tube-to-tube variation. As well as variation between tubes introduced by washing of the cells, the necessity in these experiments of dividing the culture into individual tubes several hours before RNA synthesis can be examined, leads to considerable variation in growth in different tubes.

These experiments have demonstrated that cultures of stimulated human lymphocytes may be used to advantage for examination of whether a drug has an effect on RNA synthesis independent of an effect on DNA synthesis.

Acknowledgements—We are very grateful to Dr. R. Nery and Mr. P. J. Bell for undertaking the estimations of hydroxyurea and to Miss Susan Blunt for her excellent technical assistance.
The investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council, the British Empire

Cancer Campaign for Research, and by the Public Health Service Research Grant no. CA-03188-10 from the National Cancer Institute, U.S. Public Health Service.

During this work J. Kierski was receivng a World Health Organisation Fellowship.

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