ERYTHROPOIETIN ACTION IN RAT MARROW CELL CULTURES IN COMPLETE ABSENCE OF DNA SYNTHESIS. II. LONG TERM EFFECTS ON MACROMOLECULAR SYNTHESIS.

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SUMMARY: Rat marrow cells were preincubated for 45 hours with 5.5 x 10^{-4}M sodium hexachloroiridate. This treatment abolished DNA synthesis whilst improving cell survival over that of controls. The synthesis of RNA, protein and glycoprotein continued and could be further increased by the addition of erythropoietin for up to 44 more hours. Heme synthesis also continued in the absence of DNA synthesis but could not be stimulated by erythropoietin.

INTRODUCTION

The humoral agent erythropoietin (EPO) controls the replication and differentiation of erythroid precursor cells. It produces progressive molecular changes in them which lead ultimately to the formation of mature red cells. The effects of EPO on its target cells follow a characteristic time course. Cell culture studies have shown that the influence of EPO is first manifested by an increase in RNA synthesis within 15 minutes of its addition (1). Increases in the synthesis of DNA (2), stromal glycoprotein (3) and iron uptake (4) follow after 2-4 hours. Finally, 5-10 hours after the addition of the hormone there is an increase in hemoglobin synthesis and in globin mRNA activity (5). As a result of these studies it may be assumed that EPO activates the sequential transcription of a number of different genes within the nuclei of its target cells. The delay in the onset of EPO stimulated hemoglobin synthesis in hemopoietic cell cultures

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Abbreviations: EPO, erythropoietin; Ir, sodium hexachloroiridate; TCA, trichloroacetic acid.

and the requirement for several rounds of DNA synthesis in dimethylsulfoxide stimulated Friend virus transformed cells before an increase in hemoglobin synthesis can take place (6,7) suggest the possibility of a tight coupling between DNA synthesis and the phenotypic expression of certain genes related to erythroid differentiation. In an attempt to define those molecular events which can take place in cells in response to EPO in the absence of DNA synthesis we have recently begun to culture rat marrow cells in the presence of sodium hexachloroiridate (Ir), an agent reported to arrest mammalian cells in the G_1 phase of the cell cycle (8). We have reported that we found in Ir-treated cultures a stimulation of RNA synthesis by EPO within one hour of its addition which was comparable to that obtained in control cultures (9). The present study extends these observations to later effects of EPO on RNA synthesis and to effects of EPO on protein, stromal glycoprotein and heme synthesis. The results to be reported suggest that among the effects of EPO examined only the one on heme synthesis depends on concurrent DNA synthesis.

MATERIALS AND METHODS

Rat bone marrow cell cultures were performed as described previously (9,10). DNA, RNA, protein and stromal glycoprotein synthesis was measured based on incorporation of appropriate labelled precursors into trichloroacetic acid (TCA) precipitable materials. Samples were prepared for liquid scintillation counting as described before (9). Counting efficiency for [^{3}H] and [^{14}C] was 29% and 70% respectively. Heme synthesis was determined based of [^{59}Fe] incorporation into cyclohexanone extractable material (10). Counting efficiency was 8%. In all instances cells were labelled during the last hour of culture. Nucleated cell counts were performed with a model ZBI Coulter Counter at the start and end of each culture period. When it was desired to abolish DNA synthesis cultures were preincubated for 45 hours with 5.5 x 10^{-4}M Ir. The complete elimination of DNA synthesis was always verified by monitoring [^{3}H]thymidine incorporation of Ir treated cells. Sodium hexachloroiridate (Na_2 Ir Cl_6, $6H_2O$) was purchased from Ventron Corporation Alfa Products. [^{3}H] uridine (21 Ci/mole); [^{14}C] amino acids uniformly labelled mixture, (1 mCi/mg); and [^{59}Fe] Cl_3 (25.1mCi/mg) were obtained from ICN Chemical and Radioisotope Division. [^{3}H] methylthymidine (6.7 Ci/mmole and [1-^{14}C] glucosamine (8.2mCi/mmole) were bought from New England Nuclear.

Human urinary erythropoietin (preparation H-10-TaLSL, 85.2 units/mg protein was generously supplied by the National Heart and Lung Institute. It had been collected and concentrated by the Department of Physiology, University of the Northeast, Corrientes, Argentina and was processed further by our laboratory. The concentration of EPO employed was always 0.14units/ml cell suspension.

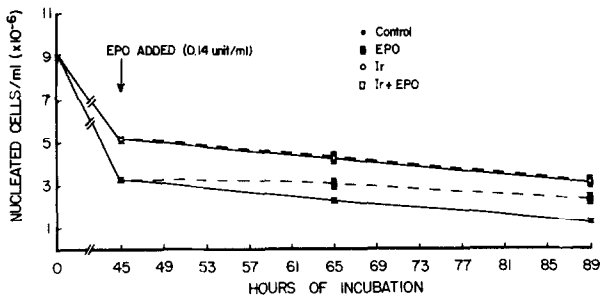

Figure 1. Influence of Ir and EPO on nucleated cell numbers Values are the means ± S.E. from 3 cell counts.

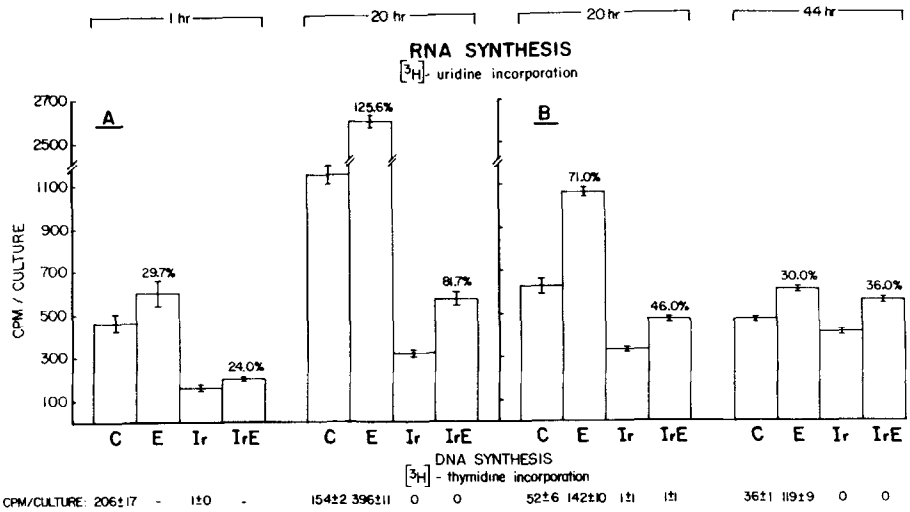

Figure 2. Effect of Ir on EPO stimulated RNA synthesis. EPO was added at the end of 45 hours and incubation continued for the indicated time. Cells were labelled with 1 μCi of [3H] uridine or [3H] thymidine per culture respectively. Values are the means ± S.E. from 3 determinations. Stimulation of incorporation by EPO over that of controls is shown as % increase.

RESULTS

Effect of Ir and EPO on nucleated cell numbers. We have reported previously (9) that 45 hours exposure to Ir, at a dose which abolished DNA synthesis, reduced the loss of nucleated cells during culture. It was found that the same also holds true for 65 and 89 hours treatment with Ir (figure 1). In the untreated cell cultures EPO significantly increased

the nucleated cell numbers over that of controls without EPO whereas Ir-treated cells failed to show any such increment due to EPO. This observation may best be explained by the blockage of cell division in the Ir-treated cultures. However, the possibility should also be considered that EPO did not stimulate cell proliferation but protected cells from lysis. In the following experiments the data on EPO stimulated radioactive incorporation are presented on a per culture rather than on a per nucleated cell basis. This was done because it seemed highly probable that the ratio of the number of nucleated cells to number of cells capable of responding to EPO varied under different culture conditions.

RNA synthesis. Figure 2 describes EPO effects on RNA synthesis in 2 representative experiments. In experiment A both cells pretreated and not pretreated with Ir showed a considerable increase in RNA synthesis within one hour of the addition of EPO as reported previously (9). After they had been exposed to EPO for 20 hours the Ir-treated cells continued to exhibit increased RNA synthesis in the absence of DNA synthesis and cell division. This pattern of an EPO effect on Ir-treated cells was also obtained 44 hours after its addition as shown in experiment B.

Protein synthesis. In preliminary experiments it was established that a stimulation of protein synthesis due to EPO could only be consistently observed 7 or more hours after its addition to the preincubated cultures. Figure 3 shows 2 representative experiments which demonstrate that, after 20 and 44 hours of exposure to EPO, protein synthesis was increased both in the presence and absence of DNA synthesis.

Constancy of precursor pool sizes in the presence and absence of EPO. It had previously been established that during the first hour after the addition of EPO, both in cultures containing and not containing Ir, the intracellular pools of RNA precursors remained unchanged (9). Utilizing the same method for the assessment of pool sizes as employed before, we found that this also held true for the relative pool sizes 20 hours after

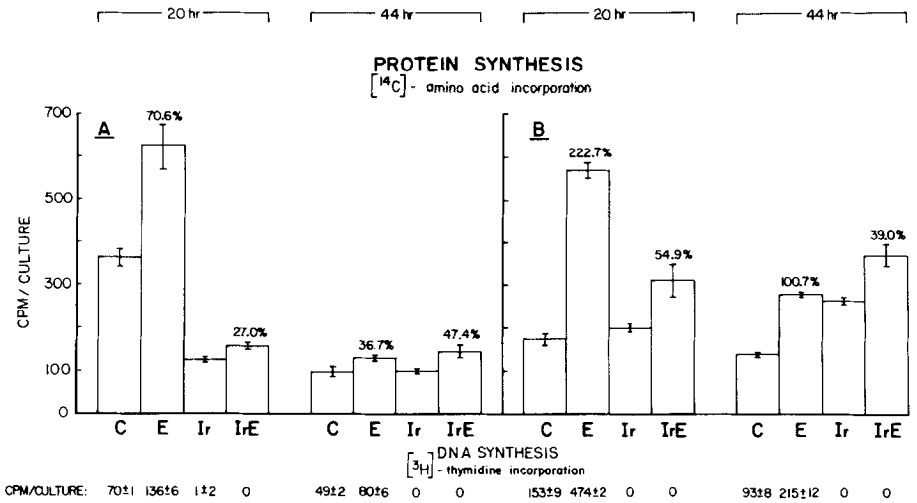

Figure 3. Effect of Ir on EPO-stimulated protein synthesis. Cells were labelled with 0.5 μCi of [14C] amino acids per culture. Other information as in figure 2.

the addition of EPO (Table 1). Amino acid pool sizes were also examined similarly. Again no changes in pool size due to EPO were found 7 and 20 hours after its addition (not shown and Table 1.).

Stromal glycoprotein synthesis. EPO preparations cause an increase of [14C] -glucosamine incorporation into acid insoluble constituents of marrow cells in culture (3). The effects of several doses of Ir on glucosamine incorporation in the absence and presence of EPO were investigated. Figure 4 shows an experiment in which preincubated cultures were continued for 6 more hours after EPO addition. An intermediate dose of Ir which inhibited 2/3 of the DNA synthesis present in the controls brought about an increase in glucosamine incorporation. A dose of Ir which inhibited all DNA synthesis however, did not cause such an increase. Large Ir doses reduced, but did not abolish, the stimulatory effect of EPO on glycoprotein synthesis which persisted even in the complete absence of DNA synthesis. After 20 hours of exposure to EPO, cells completely devoid of DNA synthesis continued to show increased glucosamine incorporation (not shown).

Table 1. Determination of Relative Precursor Pool Sizes

	CPM/Culture				Stimulation due to EPO (%)	
	Control		Iridium			
Level of Isotope (μCi/ml)	-EPO	+EPO	-EPO	+EPO	Control	Iridium
[^{3}H] uridine						
1.0	2887± 69	16908± 691	1969±60	3023±59	485.7	53.5
4.0	10326±593	59780±2185	7172±42	10918±49	478.9	52.2
[^{14}C] amino acids						
0.5	348± 2	658± 14	304± 2	447± 9	89.1	47.0
2.0	1304± 2	2500± 14	1290±12	1933± 9	91.7	49.8

EPO was added at the end of 45 hours and incubation continued for 20 hours. Incorporation values are the means ± S.E.from 3 determinations.

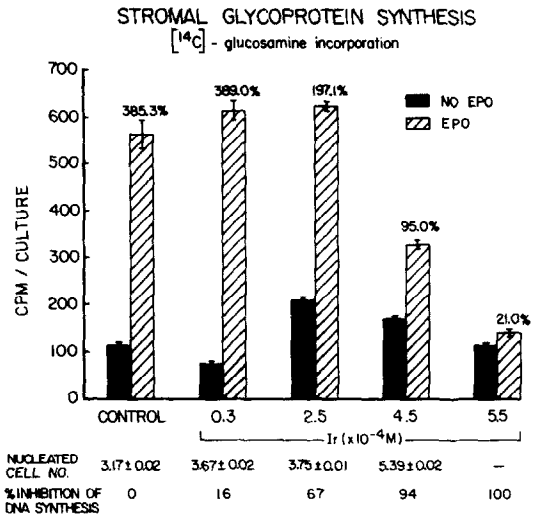

Figure 4. Effect of Ir and EPO on stromal glycoprotein synthesis. EPO was added at the end of 45 hours and incubation continued for 6 hours. Cells were labelled with 0.5 μCi of [14C] glucosamine per culture. Below the bars are shown Ir concentration, nucleated cell count/ml at the end of the culture and extent of inhibition of DNA synthesis as %. Other information as in figure 2.

Heme synthesis. Figure 5 contains data from 2 representative experiments on heme synthesis 10 and 20 hours after the addition of EPO to preincubated cells. Cultures with unimpaired DNA synthesis displayed a normal response to EPO. However, in cultures in which DNA synthesis had been abolished by Ir, it was found that although heme synthesis persisted it could not be stimulated by EPO. The same held true for heme synthesis 44 hours after EPO addition (not shown).

DISCUSSION

Preincubation of rat marrow cell cultures with Ir creates an experimental system in which DNA synthesis is selectively abolished and the cells are presumed to be arrested in the G_1 phase of the cycle. This allows one to explore which EPO stimulated cellular processes depend on DNA synthesis for their appearance and continuation and which do not. In the present communication we have shown that rat marrow cells, rendered incapable of

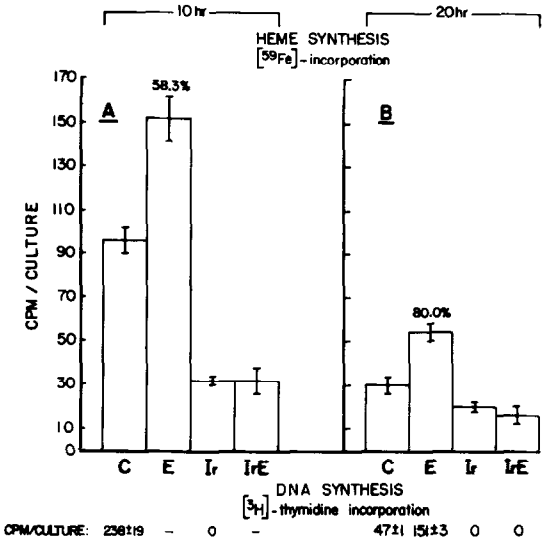

Figure 5. Effect of Ir on EPO-stimulated heme synthesis. Cells were labelled with 3 μCi of [^{59}Fe] bound to human serum proteins per culture. Other information as in figure 2.

DNA synthesis, initiate and continue for at least 2 days increased RNA and protein synthesis in response to EPO. This demonstrates that at least a certain portion of gene expression involving transcription and translation related to EPO action is possible in cells which do not synthesize DNA and cannot proceed form the G_1 into the S phase. EPO also caused an increase in glycoprotein synthesis in such cells as measured by the [^{14}C]-glucosamine incorporation system. It should be noted that this system although sensitive to EPO is not specific for it. One can, by the velocity sedimentation technique, separate out fractions from a mixture of marrow cells which respond to EPO with an increase of glucosamine incorporation but not with an increase of heme synthesis; also EPO preparations highly purified with respect to the in vivo assay were found to contain, on a weight basis, less glucosamine incorporation stimulating activity than their lower purity starting materials (M.C. Datta and P.P. Dukes, unpublished observations). This suggests that stimulation of glucosamine incorporation and therefore action on what may be

several types of hemopoietic cells by EPO and other factors is possible in the absence of DNA synthesis. The observed reduction in this stimulation with increasing Ir doses could be equally well explained by a restriction of the observable effect to that of EPO alone or by increasing degrees of alteration of the cell membrane by Ir, which may possibly also be related to the cessation of DNA synthesis (8).

We found that in Ir treated rat marrow cells heme synthesis continues but it cannot be stimulated by EPO. This would indicate a requirement by this process for concurrent DNA synthesis similar to that which has been postulated for fetal mouse liver cell cultures (11). Several authors have also suggested that induction of hemoglobin synthesis in Friend leukemia cells grown in culture has a similar requirement (6,7). Recently, however, Leder et al. (12) reported that when they treated such cells with butyric acid instead of dimethysulfoxide, as was done previously, they were able to induce hemoglobin synthesis in the absence of DNA synthesis or cell division. Therefore, the generality of the requirement of prior DNA synthesis for the induction of hemoglobin synthesis (transcription or unmasking of hemoglobin mRNA?) remains open to question.

The ability of Ir-treated rat marrow cells to respond to EPO with increases in RNA and protein production will now be further exploited by an investigation of the nature of differences between these macromolecules synthesized in the absence and presence of DNA synthesis with the hope that this will provide information on "early" events in erythroid differentiation.

ACKNOWLEDGEMENTS

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