of the degree of its affinity for protein, can act as substrate, within the limits of sensitivity of the method and under the experimental conditions used, for the presumptive oxidoreductase activity of UEBP.

The results are further confirmation of the regulatory function of UEBP, realized through temporary exclusion of protein ligands from the general hormonal biodynamics. The fact that UEBP is found in the soluble fraction of cells rules out the likelihood of mono-oxygenase activity of this protein [2]. Meanwhile there remains the possibility that UEBP possesses a different type of enzyme activity (transferase, for example), relative to its ligands.

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EFFECT OF INHIBITORS OF POLYAMINE BIOSYNTHESIS AND OF EXOGENOUS POLYAMINES ON MITOGENIC EFFECT OF EPIDERMAL GROWTH FACTOR IN PRIMARY HEPATOCYTE CULTURE

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Activity of the enzymes of polyamine biosynthesis correlates positively with the proliferation rate of cells. It has been shown in the case of the regenerating liver that stimulation of proliferative processes is accompanied by increased activity of the key enzyme of polyamine synthesis, namely ornithine decarboxylase (ODC), during the hours immediately after the operation [4]. Temporary inhibition of ODC prevents the cells of the regenerating liver from entering the S phase of the mitotic cycle [9]. However, contradictory results have been obtained on primary cultures of hepatocytes [7, 8]. It has been shown, in particular, that several hormones possess a stimulating action on ODC, including some which are not inducers of cell proliferation [6]. There are likewise conflicting data on the effect of amines on proliferative processes, although it is known that an increase in the intensity of polyamine

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synthesis is one response of the cell to the action of polypeptide growth factors and, in particular, of epidermal growth factors (EGF) [5].

The aim of this investigation was to study the role of polyamines in the realization of the mitogenic effect of EGF in primary hepatocyte culture.

EXPERIMENTAL METHOD

Isolated liver cells were obtained and a primary monolayer hepatocyte culture set up as described previously [1, 4]. The medium was first changed 18-20 h after application of the cells to trays measuring 6 × 3 cm^2 ($75 \cdot 10^4$ hepatocytes per well) or coverslips ($6\text{-}7 \cdot 10^4$ cells per coverslip), for which purpose the cells were washed 3 times with Hanks' solution, after which fresh serum-free Eagle's medium and the corresponding components were added. The concentration of EGF was 0.5-5 nM, of the inhibitors of polyamine biosynthesis difluoromethylornithine (DMFO) – 10^{-3} M, and methylglyoxal-bis-(guanylhydrazone) (MGBH) – 10^{-4} M, and of putrescine, spermine, or spermidine – 10^{-8} M. After 20 h, ^{3}H-methylthymidine was added to the cultures in an amount of 74 kBq/ml of medium, and 2 h later, cells growing on coverslips were used to prepare cytoautoradiographs, as described previously [4]. In hepatocytes growing on the bottom of the trays, incorporation of label into DNA was determined 4-6 h after addition of ^{3}H-methylthymidine, for which purpose the residue insoluble in 5% TCA was applied to nitrocellulose filters. The radioactivity of the residues was determined in toluene scintillator ZhS-7 on a "Beckman LS-180" counter (USA). Binding of EGF with the hepatocytes was determined quantitatively on the 2nd day of culture, for which purpose polyamines and ^{125}I-EGF (New England Nuclear, USA) were added to the trays 4 h after addition of the inhibitors of polyamine synthesis, in a quantity of 600,000 cpm, after which the trays were incubated for a further 90 min. The cells were then washed 6 or 7 times with an excess of Hanks' solution, lysed overnight in 0.5 M NaOH, after which the radioactivity of the lysates was determined on a "Gamma-1" counter (USSR). The level of nonspecific binding of ^{125}I-EGF was determined after preliminary incubation of the hepatocytes with an excess of unlabeled EGF, isolated from the salivary glands of mice as described previously [2].

EXPERIMENTAL RESULTS

The study of the mitogenic effect of EGF in primary hepatocyte culture showed that the largest number of S-phase hepatocytes was present 46-48 h after addition of the mitogen, and the level of the proliferative response of the hepatocytes was inversely proportional to the age of the rats donating the liver cells. For instance, in a culture of hepatocytes isolated from the liver of unweaned rats, the mitotic index was 1.72% as early as 24 h after addition of EGF, or almost 10 times higher than in a culture of hepatocytes from adult rats. Addition of spermine, but not of putrescine or spermidine, to serum-free medium led to a very small increase (by 20-25%) in incorporation of ^{3}H-methylthymidine into the cell DNA. The study of the effect of inhibitors of polyamine biosynthesis on realization of the mitogenic effect of EGF in hepatocyte culture showed that both preliminary (6 h before administration of EGF) and simultaneous addition with EGF of each of the inhibitors individually or of all of them together, prevents stimulation of DNA synthesis induced by EGF (Table 1). The most marked inhibitory properties were exhibited by a mixture of the two inhibitors DFMO and MGBH, the use of which reduced incorporation of the label into hepatocytes by more than 70%. According to the autoradiographic data, blocking of entry of the hepatocytes into the S phase of the mitotic cycle was even more marked, at 95-97%. The differences were probably due to the fact that to determine incorporation of the radioactive label, cell residues were used and not DNA preparations isolated from them. The known cytotoxicity of MGBH also must be mentioned [11]. Besides vacuolation of the cytoplasm and shrinking of the cells, the density of the culture was reduced and a large proportion of hepatocytes was transferred into suspension by the 3rd day.

Inhibition of activity of the key enzymes of polyamine biosynthesis thus prevents hepatocytes from entering the S phase of the mitotic cycle. Reduction of the endogenous pool of polyamines directly involved in the basic processes of cell metabolism, the intensification of which is observed in the G_1-phase, and which is essential for maintaining DNA replication, probably inhibits realization of the mitogenic effect of EGF. However, the probability likewise cannot be ruled out that inhibitors of polyamine biosynthesis can directly or indirectly affect binding of EGF with specific receptors and internalization of the ligand-receptor complex. The results of experiments in which polyamines were added to the he-

TABLE 1. Inhibition of EGF-Stimulated DNA Synthesis in Hepatocytes by Inhibitors of Polyamine Biosynthesis (M ± m)

Version of experiment	Mitotic index, %	Incorporation of ^{3}H-thymidine into DNA, cpm/dish	
		2nd day of culture	3rd day of culture
1 nM EGF	32,7±4,2	25 700	13 100
1 nm EGF + DFMO	6,8±2,3	9 100 (64,5)	6 070
1 nm EGF + MGBH	0,9±0,4	8 300 (67,8)	4 700
1 nm EGF + DMFO + MGBH	0,6±0,2	6 850 (73,5)	4 580
1 nm EGF + concanavalin A	—	11 400 (55,7)	—
Control (medium without serum)	6,9±1,1	7 200	3 850

Legend. Inhibition of EGF-stimulated incorporation of label (in %).

TABLE 2. Effect of Inhibitors of Polyamine Synthesis and of Polyamines on Binding of ^{125}I-EGF with Hepatocytes (in cpm/dish)

Inhibitor	Control	Putrescine	Spermidine	Spermine
DFMO	4550	4430 (++)	4170 (++)	4030 (+++)
MGBH	3870	3510 (+)	2380 (+)	4800 (+)
DFM+ + MGBH	4480	3320 (+)	4010 (+++)	4250 (++)
Control (medium without serum)	3210	3780	2470	4150

Legend. Effectiveness of restoration of DNA synthesis by polyamines against the background of inhibition by inhibitors shown in parentheses: +) partial ++) virtually complete, +++) in excess of that induced by EGF.

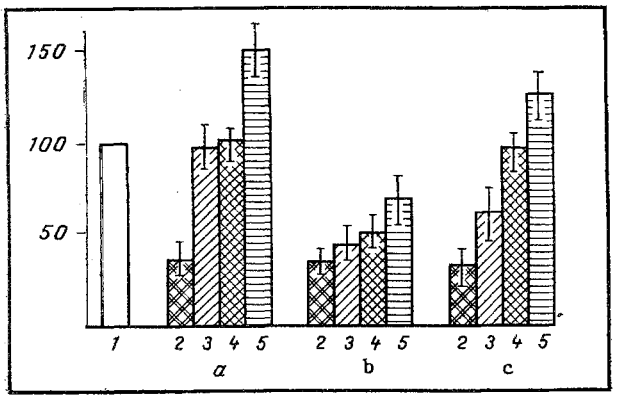

Fig. 1. Restoration of incorporation of ^{3}H-methylthymidine into hepatocyte DNA, blocked by polyamine inhibitors, by putrescine (3), sperimidine (4), and spermine (5). Level of incorporation in presence of 1 nM EGF (1); 1 nM EGF + DFMO (a); 1 nM EGF + MGBH (b); 1 nM EGF + DFMO + MGBH (c); 2) control.

patocyte culture, shown in Fig. 1, showed that all the polyamines used equally restored DNA synthesis in the presence of DMFO. Spermine, moreover, potentiated the mitogenic effect of EGF. However, none of the polyamines had a positive effect on DNA synthesis against the background of the action of MGBH, most probably because of the marked toxic action of the latter. Nevertheless, when DNA synthesis was inhibited by a mixture of inhibitors, the mitogenic ac-

tion of EGF was restored by the addition of spermidine or spermine. A similar effect of natural polyamines, reported previously [13], differs from that of certain exogenous, including lysosotropic, amines, which also block mitogenic stimulation of cells induced by EGF [10, 11]. The results of experiments to determine binding of ^{125}I-EGF with hepatocytes (Table 2) show that inhibitors of polyamine synthesis not only do not reduce binding, but to a certain extent increase binding of the growth factor with the cells. DFMO has a stronger action, and in its presence, binding of EGF with hepatocytes was increased by 20-25%. The addition of polyamines to the culture medium likewise had no significant effect on interaction of ^{125}I-EGF with liver cells. No correlation could be found between the quantitative change in binding of EGF with hepatocytes and the level of restoration of the blocked DNA synthesis on the addition of polyamines. The preliminary results support the view that the inhibitors and polyamines tested have no significant effect on the internalization of the EGF-receptor complex. Meanwhile concanavalin A, preventing endocytosis and internalization of this complex [12], blocks the mitogenic effect of EGF in hepatocyte culture (Table 1).

Thus both the inhibitory action of DFMO and MGBH on realization of the mitogenic effect of EGF and prevention of their effect by exogenous polyamines are not connected with disturbance of interaction of growth factor with the cells, but are due to the influence of the test substances on processes of intracellular biosynthesis in cells stimulated toward proliferation.

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