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SURFACE ORIGIN OF ERYTHROCYTIC CHALONE in vitro IN EXPERIMENTAL POLYCYTHEMIA

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Tests on surviving cultures of bone marrow cells from rats with experimental polycythemia showed that the chalone activity of erythrocytic chalone is considerably reduced in the presence of phytohemagglutinin (PHA). The chalone depresses the agglutinating activity of PHA on bone marrow cells. Adsorption of the chalone preparation on immobilized PHA leads to disappearance of electrophoretically recorded PAS-positive bands from it and a sharp decrease in the intensity of the PAS-negative band. Experiments with preliminary incubation on rat erythrocytes before isolation of the chalone preparation suggest that in the course of its isolation two polypeptides are selectively liberated into the medium, one of them being PAS-positive. It is suggested that erythrocyte surface membrane proteins are components of the chalone. The possible mechanism of liberation of chalones from the surface of the cells and the particular features of their action on cells are discussed.

KEY WORDS: erythrocytic chalone; bone marrow cells.

Much attention is currently being paid to chalones, endogenous regulators of cell division [4, 5, 12]. The suggestion that they may be components of the surface of normal cells appears helpful toward the understanding both of the character of their liberation and the mechanism of their action.

The results of an investigation of erythrocytic chalone are described below.

EXPERIMENTAL METHOD

A surviving culture of albino rat bone marrow cells [2] was used. The "polycythemia in vitro" model [1] was used to obtain erythrocytic chalone (EC).

Before incubation the erythrocytes were washed 3 times with physiological saline in order to rule out any possible contamination by serum proteins.

As proof of the membrane origin of EC their ability to bind with phytohemagglutinin (PHA) was used. It was assumed that if EC contains PHA receptors, its inhibitory activity toward bone marrow cells ought to be reduced in the presence of lectin. Conversely, on the addition of N-acetyl-D-galactosamine (NAGA) to the PHA + EC complex the inhibitory effect ought to increase because of the blocking of PHA by specific hapten.

To test these hypotheses the following series of experiments were carried out: 1) control – intact cells, 2) cells with the addition of NAGA, 3) cells with PHA, 4) cells with EC, 5) cells with PHA + EC, 6) cells with PHA + EC + NAGA. The PHA concentration in the experiments varied from 10 to 400 μg/ml medium. The concentrations of the other substances are given in the caption to Fig. 2. Colchicine was added to the Eagle's medium with the cells 15 min before incubation began. The bone marrow cells were fixed 2 h later and stained by the Romanovsky–Giemsa method. To estimate the mitotic activity of the erythron [1] the stathmokinetic index among the proliferating cells was calculated. The numerical results were subjected to statistical analysis by Student's test. When high concentrations of PHA (400 μg/ml) were used the character of action of the various factors was judged by the "delay of agglutination" criterion, which was determined by counting the number of agglutinates formed in 20 fields of vision of the microscope (magnification 900×).

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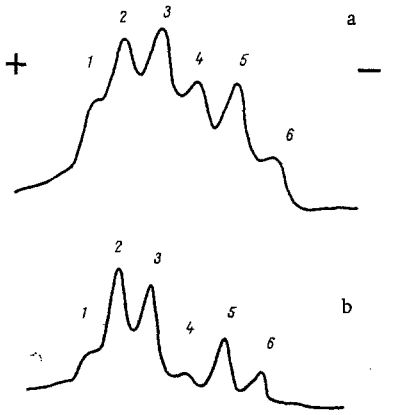

Fig. 1. Densitograms of gels after disc electrophoresis of erythrocyte ghost supernatant after incubation for 1 h. a) Ghosts of control erythrocytes (without preincubation); b) erythrocyte ghosts after preincubation of cells for 1 h. Gel No. 1 system, electrophoresis at 2°C, current 1.8 mA/gel, stained with Coomassie. 1-6) Nos. of fractions.

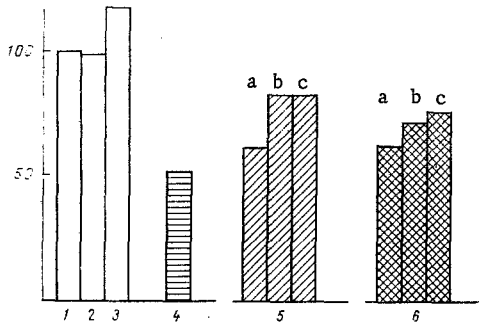

Fig. 2. Mitotic activity (c-mitoses) of cells of erythroid series. 1) Intact cells; 2) after action of NAGA (70 μg/ml); 3) PHA (10-80 μg/ml); 4) EC (0.6 mg/ml); 5) PHA + EC; 6) PHA + NAGA + EC. PHA concentration in experiments 5 and 6 for a, b, and c was 10, 40, and 80 μg/ml respectively. Difference between experiments 2, 3, and 1, between 4 and 5a, and between 5 and 6 not significant ($P > 0.05$); difference between experiments 1 and 4, and 4 and 5b significant ($P < 0.05$). Abscissa, variants of experiments; ordinate, mitotic activity (in % of control – experiment 1).

To study whether erythrocyte membrane proteins are liberated into the incubation medium, experiments were carried out with erythrocyte ghosts before and after preliminary incubation. Erythrocytes washed to remove plasma proteins were divided into two parts, from each of which ghosts were obtained, the difference being that one batch of erythrocytes was preincubated at 37°C in Hanks' medium for 1 h. The ghosts obtained from the two groups were then incubated for 1 h at 37°C in Hanks' medium, and after centrifugation the supernatant was fractionated by electrophoresis in polyacrylamide gel under the conditions described in [1]. Electrophoretic fractionation of the supernatant of the erythrocyte ghosts after incubation for different times (1 and 2 h) was thus compared.

To study binding of EC with PHA the following experiments were carried out: 1) electrophoresis of the PHA + EC combination, 2) adsorption of the test erythrocyte extracts on PHA cross-linked with porous glass.*

*The preparation of cross-linked PHA was generously provided by N. V. Golovina (Mikrobioprom), to whom the writers are grateful.

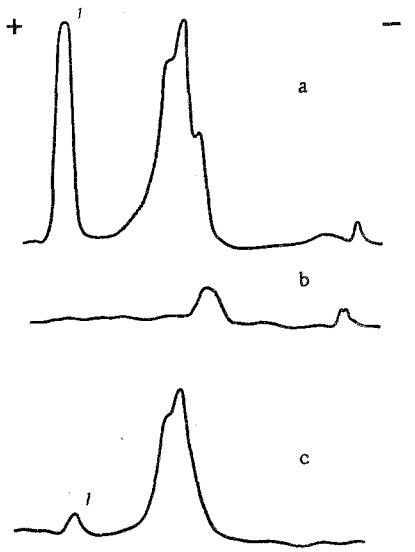

Fig. 3. Densitograms of gels after disc electrophoresis of complete (a, b; without adsorption on PHA) and incomplete erythrocyte extract (c; after adsorption on PHA). a, c) Stained with Coomassie, b) stained by Schiff's method.

Altogether 56 rats weighing 120-150 g were used. The sources of the reagents were as follows: the kit of chemical reagents for disc electrophoresis and the PHA from Reanal, Hungary; Eagle's medium from Staatliches Institut für Immunpräparate und Nährmedien, East Germany; colchicine from Merck, West Germany; NAGA from BDH Chemicals, England.

EXPERIMENTAL RESULTS

Comparison of the results of electrophoresis of the supernatant of erythrocyte ghosts incubated for different times (Fig. 1) shows that two proteins, 1 and 4, of which one (4) is PAS-positive, are selectively liberated into the incubation medium. PHA receptors are known [9] to be PAS-positive proteins. If the erythrocyte extracts in fact contained erythrocyte membrane proteins, in the presence of PHA their electrophoretic mobility should have been changed, but this was not observed experimentally. This fact may indicate that in an alkaline medium (electrophoresis at pH 8.9) the PHA + EC complex dissociates, whereas in a neutral medium this evidently does not happen. This suggestion was confirmed by the results of experiments using hemagglutinating concentrations of PHA, namely 400 μg/ml, causing 100% agglutination of bone marrow cells, which made it impossible to calculate the mitotic index (number of c-mitoses 0%). If the PHA was added in these experiments in the presence of EC, agglutination was inhibited by 16%, but if the PHA + EC + NAGA complex was added, agglutination was inhibited by 29%. The mitotic activity among the unagglutinated cells in these experiments was 6 and 8% respectively.

Further confirmation of the formation of a PHA + EC complex in neutral medium was given by the results of tests with incubation of bone marrow cells (Fig. 2) and data on adsorption of the EC preparation on immobilized PHA (Fig. 3). If PHA receptors are components of EC, the chalone activity of the preparation ought to be reduced in the presence of PHA. If, however, no such receptors are present in the EC molecule, the chalone activity of the preparation should not change significantly. As the results in Fig. 2 show, the chalone activity of the PHA + EC complex was lower than that of EC alone (columns 5 and 4 respectively). A similar picture was observed when cells were treated with the PHA + NAGA + EC complex (column 6). It follows from the results of these experiments that NAGA has a weak effect on the chalone activity of EC in the presence of PHA. This result may perhaps be explained by competition between EC and NAGA for the combining sites on the PHA molecule. However, it cannot be finally concluded from these results that PHA receptors in the EC preparation possess chalone activity.

Adsorption of the EC preparation on immobilized PHA led (Fig. 3) to disappearances of the PAS-positive bands in the EC preparation and to a marked decrease in the intensity of the PAS-negative band 1. Disap-

pearance of the PAS-positive proteins was due to their direct binding with lectin, whereas the sharp decrease in the PAS-negative fraction 1 in the extracts was evidently caused by its interaction with the complex formed between PHA and the PAS-positive protein. These results suggest that both proteins (PAS-positive and PAS-negative) were responsible for the chalone effect. Alternatively, the chalone activity of the preparation could be connected with the action of one protein, whereas the other was the cell receptor for it.

Comparison of the chalone activity of the EC preparation after adsorption on immobilized PHA with that of the EC preparation without adsorption on PHA, by calculating the mitotic index of the bone marrow cells, showed a decrease in the activity of the preparation by 78%. These findings correlate well with experiments on bone marrow cells (Fig. 2). Whatever the interpretation of the results, it is impossible to attribute the activity of EC entirely to PAS-positive proteins of the erythrocytes or the PAS-negative fraction 1.

In the presence of PHA binding of the PAS-positve proteins and PAS-negative fraction 1 of the erythrocyte extracts thus took place; the PHA + EC complex or the EC preparation itself after adsorption on immobilized PHA had reduced chalone activity compared with complete EC; the decrease in the chalone activity of EC was accompanied by disappearance of PAS-positive proteins and the PAS-negative fraction from the erythrocyte extracts; on the basis of the ability of erythrocyte proteins to bind with PHA and also on the differences the rate at which individual fractions of erythrocyte ghost proteins enter the incubation medium, it can tentatively be suggested that the chalone activity of the erythrocyte extracts is due to surface membrane proteins.

These results, of course, are not final proof of the surface origin of EC, but they enable this to be put forward as a working hypothesis.

Within the framework of this hypothesis the writers suggest that liberation of erythrocyte surface proteins is the result of activity of membrane-bound proteases, which are found in erythrocyte membranes and whose activity is partly inhibited by Ca^{++} [13].

The view that chalones are surface proteins of erythrocyte membranes, liberated by endogenous membrane proteases, is in agreement with the increased blood level of orosomucoid (sialoglycoprotein) of tumor-bearing animals [3] and with the appearance of sialoglycoproteins in the fluid of ascites tumors [10]. In fact, transformed cells have higher endogenous membrane protease activity than normal cells [7, 11]. It may be chalones, if not liberated from membranes, have a suppressive action on the host cell, inhibiting its mitotic activity. Liberation of chalones from the plasma membrane of the cell inhibits mitotic activity of the cells with whose plasma membrane they have just interacted. This may perhaps explain the sigmoid character of tumor development: In the stage of progression of the tumor, chalones liberated intensively from malignant cells have a regressive action on its development. Factors capable of liberating chalones, such as trypsinization, or proteins which can compete with endogenous membrane chalones for specific sites on the plasma membrane, namely serum proteins or mitogenic lectins, according to this hypothesis ought to have, and indeed, do have, a mitogenic action.

On this basis the writers suggest that cellular homeostasis at the organism level may be achieved within cells and tissues by the quantity of endogenous chalones on the cell surface and by factors influencing their liberation (membrane proteases) and, possibly, by surface sialoglycoproteins, which either fix the chalones on the plasma membrane through steric hindrances to their liberation or inhibit the activity of membrane-bound proteases, as a result of their ability to bind Ca^{++} [8]. Possible confirmation of this role of sialoglycoproteins is given by their inhibitory action on stimulation of lymphocytes observed in a mixed culture, which is inhibited as a result of their preliminary treatment with neuraminidase [6], and also data showing an increase in the number of sialic acid residues on the surface of cells in the course of their maturation.

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MULTISTAGE REGULATORY SYSTEM TRANSMITTING THE ACTION OF ESTRADIOL IN THE RAT UTERUS

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Estradiol induces histidine decarboxylase in the tissues of the uterus, and histamine activates adenylate cyclase. Cyclic AMP, like histamine, stimulates the action of estradiol by stimulating RNA synthesis and inducing enzymes of glycolysis and hydration of the uterus. Autoradiography showed that [^{3}H]estradiol is accepted by the nuclei of some myometrial cells, and [^{3}H]histamine by the cytoplasm of these cells; [^{3}H]cyclic AMP is selectively bound by the endothelial cells of the uterine capillaries. Estradiol mediators (histamine and cyclic AMP) evidently spread the effect of the hormone to heterofunctional cells of the uterus which together constitute a unique type of multicellular functional assemblage.

KEY WORDS: Estradiol; histamine; cyclic AMP; induction.

Investigations have shown that histamine and cyclic AMP simulate the principal physiological effects of estradiol: hyperemia, hydration of the uterus, etc. [6, 12, 14]. It was shown recently in the writers' laboratory that histamine and cyclic AMP are mediators in the action of pentagastrin. Together with certain enzymes, they constitute a unique type of amplification cascade, spreading the action of the hormone to various adjacent cells forming a multicellular functional assemblage [10]. It might be supposed that this principle of regulation is used in several systems of multicellular organisms and that, in particular, a similar amplification cascade of enzymes and mediators, including histamine and cyclic AMP, transmits the action of estradiol. The investigation described below was carried out in order to test these hypotheses.

EXPERIMENTAL METHOD

Sexually mature female Wistar albino rats weighing 140-160 g were used and ovariectomized bilaterally three weeks before the experiment. Each variant of the experiment was carried out on three or four animals. Estradiol-17β (10 μg/100 g), histamine·2HCl (250 μg/100 g), and cyclic AMP (10 mg/100 g) with theophylline (10 mg/100 g) were injected intraperitoneally. Actinomycin D was injected 15 min before the test substances. [^{14}C]Uridine (specific activity 13.9 μCi/mmole) was injected intraperitoneally in a dose of 20 μCi/100 g body weight 1 h before sacrifice. RNA synthesis was determined from incorporation of [^{14}C]uridine into the acid-insoluble fraction of uterine tissue [5]. Hexokinase and pyruvate-kinase activity was determined by Singhal's method [12]. Histamine-decarboxylase activity, detectable in segments of uterus, was judged from the quantity of [^{14}C]histamine formed from [^{14}C]histadine [11]. Adenylate cyclase activity in uterine tissue homogenates was determined by the method of Krishna et al. [7] and the DNA concentration in the tissues by the method of Dishe and Rosenfeld [3]. The distribution of ^{3}H-labeled estradiol, histamine, and cyclic AMP in the uterine tissues was investigated by autoradiography. Segments of uterus were incubated for 30 min in the presence of 10 μCi/ml [^{3}H]estradiol (specific activity 9.3 Ci/mmole; USSR), [^{3}H]histamine (specific activity 9.4 Ci/mmole; Radiochemical Centre, Amersham, England), or [^{3}H]cyclic AMP (specific activity 20.7 Ci/mmole; Radiochemi-

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