EFFECT OF LIPOXIN B ON COLONY-FORMING ABILITY OF HUMAN PERIPHERAL BLOOD MONONUCLEARS IN A DIFFUSION CHAMBER

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Proliferation and differentiation of committed precursors of granulomonocytopoiesis are absolutely dependent on contact between these cells and specific colony-stimulating factors (CSF) for each type of cell. This contact is effected by interaction between CSF and specific receptors on the surface of the precursor cells, as a result of which granulocytic, monocytic, and mixed colonies develop. Interaction of CSF with receptors leads to changes in metabolism of precursor cells of granulo-monocytopoiesis and, in particular, to an increase in tyrosine kinase activity [4].

Investigations revealing stimulation of colony formation from precursors of granulomonocytopoiesis under the influence of lipoxygenase metabolites of arachidonic acid has recently attracted attention [7, 9].

The aim of the investigation described below was accordingly to study the effect of lipoxin B and its metabolic precursor — 5,15-dihydroxy-6,8,11,13-eicosatetraenoate (5,15-DHETE) on proliferation and differentiation of granulocytic and monocytic colony-forming units (GM-CFUdc) in diffusion chambers.

EXPERIMENTAL METHOD

Blood from first-time male blood donors aged 20-30 years, collected under sterile conditions into a tube with Alsevier' solution in the ratio of 1:1 was used. After centrifugation for 30 min at 900g the supernatant was layered above a mixture of Ficoll 400 and Verografin (density 1.077) and centrifuged for 5 min at 900g. The fraction of monoclears was transferred to a plastic Petri dish to separate the adherent cells. Nonadherent mononuclears were serially diluted in medium 199 to a concentration of 1×10^6 cells/ml and then with 0.3% agarose to a concentration of 5×10^5 cells/ml, and after incubation for 30 min with the test preparations, they were introduced into diffusion microchambers of our own design. The microchamber consisted of a capillary tube 2 mm in diameter, 10 mm long, and 7 µl in volume, made from a "Millipore" (USA) filter with pore diameter 0.22 µ. The ends of the capillary tube were hermetically sealed beforehand with BF-6 glue. Microchambers filled with mononuclears were introduced into the peritoneal cavity of CBA mice by means of a special needle, the diameter of which enabled the capillary tube to be inserted by means of a stilet. The mice were given an intraperitoneal injection of cyclophosphamide in a dose of 200 mg/kg 24 h before the experiment. The recipient mice were killed 7 days later and the microchambers removed from the peritoneal cavity. One end of the microchamber was cut off with a razor blade, the contents were expelled on to a slide, and a thin film was prepared, fixed with methanol, and stained by the Romanovsky-Giemsa method. During microscopic examination of the films (40 × objective, 3 × ocular) the number of clusters was counted: up to 50 cells (small), up to 100 cells (large), and from 100 to several thousand cells (giant) colonies, with simultaneous qualitative subdivision of the colonies into granulocytic, macrophagal, mixed, and undifferentiated.

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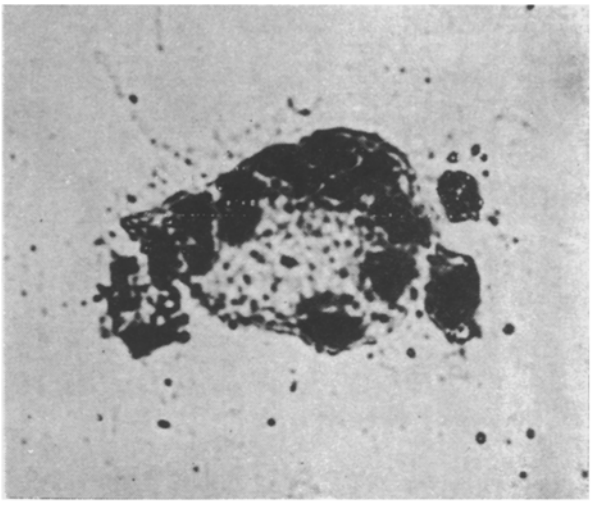

Fig. 1. Multinuclear cell formed under the influence of lipoxin B.

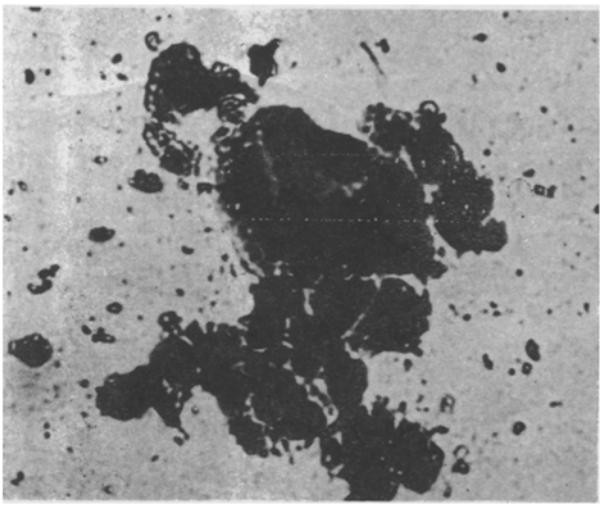

Fig. 2. Multinuclear cell at periphery of cluster formed through the action of lipoxin B.

The original solutions of the preparations in ethanol were diluted with physiological saline so that the final ethanol concentration did not exceed 1%. The action of ethanol in a dilution corresponding to that in the test preparation served as the control. The test preparations were incubated with mononuclears immediately after dilution of the original solution of the preparations.

Biosynthesis of 5,15-DHETE was carried out by catalysis of oxidation of 15-HETE, and of lipoxin B (5,14,15-trihydroxy-6,8,10,12-eicosatetraenoate) by catalysis of oxidation of 5,15-DHETE by a homogeneous preparation of 15-lipoxygenase from rabbit reticulocytes (15-LOX), obtained by the method in [17], followed by reduction of the hydroperoxide group by $NaBH_4$ and preparative isolation by high-performance liquid chromatography (HPLC) on a normal phase (Lichrosorb SI 60-10 column, 250 × 9.8 mm) in a solvent system of hexane—isopropanol—acetic acid (80:20:1) with a flow rate of 3 ml/min. The elution time of the 5,15-DHETE was approximately 8.2 min and of lipoxin B 14.5 min. The UV spectrum of lipoxin B had characteristic absorption maxima at 286, 301, and 316 nm [5]. The structure of 5,15-DHETE was demonstrated by gas chromatography mass spectrometry (GCMS).

EXPERIMENTAL RESULTS

All preparations tested had a stimulating action on colony-forming activity of the peripheral blood mononuclears. The most active preparation was lipoxin B, which had a statistically significant action in concentrations of 3.6×10^{-8}-3.6×10^{-9} M. 5,15-DHETE and arachidonic acid were less active and had a statistically significant action in concentrations of 2.5×10^{-6} M and 1×10^{-3} M respectively. Morphological analysis of the colonies formed showed predominance chiefly of macrophagal and undifferentiated colonies. The formation of multinuclear cells, located both singly (Fig. 1) and at the periphery of the colonies and clusters (Fig. 2), under the influence of the test compounds also was observed. The numerical data on the effect of lipoxin B, 5,15-DHETE, and arachidonic acid on colony formation are given in Table 1, which includes results differing statistically significantly from the control ($p<0.05$).

The results of these experiments suggest that lipoxygenase metabolites of arachidonic acid mediate the action of CSF on precursor cells of the erythroid [7] and lymphoid [3] series. This points to the existence of a common principle in the action of specific stimulators of the different branches on different hematopoietic precursors, manifested as a repeating sequence of events in the membrane and cytoplasm of the hematopoietic precursor cells after interaction of stimulators with specific receptors. It can be tentatively suggested that a mandatory stage in this sequence of events is the formation of lipoxygenase metabolites of arachidonic acid, which initiate proliferation and differentiation of hematopoietic precursor cells. Probably various lipoxygenase metabolites of arachidonic acid exert a significant influence on differentiation of hematopoietic stem cells. For instance, the action of leukotrienes C_4 and D_4 is essential for differentiation and proliferation of precursor cells of granulo-monocytopoiesis and leukotriene B_4 for the development of lymphoid colonies [3, 9]. Blockade of the lipoxygenase pathway of arachidonic acid metabolism by nordihydroguaiaretic acid and BW 755C led to

TABLE 1. Changes in Colony-Forming Activity of Human Peripheral Blood Mononuclears under the Influence of Lipoxin B, 15-DHETE, and Arachidonic Acid (M ± m)

Preparation	Clusters	Colonies								Multinuclear cells
		total	distribution by size			morphology				
			small	large	giant	macrophagal	granulocytic	mixed	undifferentiated	
Lipoxin B, concentration $3.6 \cdot 10^{-8}$ M ($n=3$)	64,33±8,51	25,0±10,54	18,33±7,36	6,67±3,18	0	10,33±2,96	0	1±1	13,67±6,89	37,67±4,09
Lipoxin B, concentration $3.6 \cdot 10^{-9}$ M ($n=3$)	50,66±11,26	21,67±6,36	16,0±4,51	5,33±1,76	0,33±0,33	6,33±2,33	1±1	2±1,54	1,67±2,60	27,67±6,44
5,15-DHETE, concentration $2,5 \cdot 10^{-6}$ M ($n=8$)	23,38±2,24	5,75±1,08	4,5±0,73	1,13±0,61	0,12±0,12	3,25±0,77	0,75±0,41	0	1,63±0,73	8,0±3,0
Arachidonic acid, concentration $1 \cdot 10^{-3}$ M ($n=6$)	19,33±1,56	4,1±1,09	3,67±0,95	0,33±0,21	0	3,33±1,17	0	0	0,67±0,49	5,67±1,38
Mononuclears from blood donors (n = 19)	8,11±1,08	1,58±0,32	1,42±0,28	0,16±0,03	0	1,21±0,26	0,11±0,02	0,11±0,02	0,16±0,03	0

Legend. Values calculated for one capillary tube.

inhibition of colony growth induced by CSF [7,9]. Preliminary incubation of human eosinophils with recombinant granulocytic-macrophagal CSF after addition of the calcium ionophore A23187 led to stimulation of leukotriene C_4 by eosinophils [10].

An extremely important item in the discussion of involvement of lipoxin B and 5,15-DHETE in the suggested chain of biochemical reactions after interaction between CSF and specific receptors on the surface of hematopoietic precursor cells is how these substances, which we added to a suspension of mononuclears in culture, penetrate into the cell from the extracellular space. This can be explained, first, by the presence of receptors specific for lipoxygenase metabolites of arachidonic acid on the surface of the blood cells [6]; the presence of similar receptors may also be postulated, moreover, on the membrane of hematopoietic precursor cells. Second, it may be taken as proven that exogenously added lipoxygenase and cyclo-oxygenase arachidonic acid metabolites are ingested by macrophages and subsequently released from the macrophages under the influence of the calcium ionophore A23187 [2].

Under the present experimental conditions, requiring 7 days for colony formation, exogenously administered lipoxygenase metabolites of arachidonic acid could be internalized initially into committed hematopoietic precursor cells, and then incorporated into the consecutive chain of biochemical events in these cells under the influence of CSF. This can also explain the active effect of the test preparations on the nuclear membrane. Multinuclear cell formation which we observed was probably the result of active processes in the nuclear membrane without the final process of cytotomy in mitosis. It can be tentatively suggested that lipoxin B facilitates disturbance of the system of actin microfilaments of the cytoskeleton in the same way as cytochalasin B [1], more especially because of data indicating that human peripheral blood monocytes can generate lipoxygenase metabolites under the influence of cytochalasin B [8].

The phenomenon of enhanced granulocytic-monocytic colony formation under the influence of lipoxin B can thus be represented as the effect of intracellular processes in hematopoietic precursor cells, induced by CSF and mediated by lipoxygenase metabolites of arachidonic acid.

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