Vol. 192, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1993 Pages 167-173

INTERLEUKIN 3 PROMOTES HISTAMINE SYNTHESIS IN HEMATOPOIETIC PROGENITORS BY INCREASING HISTIDINE DECARBOXYLASE mRNA EXPRESSION

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Received March 2, 1993

Interleukin 3 (IL-3) is a potent stimulator of histamine production by cells from murine hematopoietic organs. We demonstrate herein that this phenomenon results from increased histidine decarboxylase (HDC : EC 4.1.1.22) activity in progenitor-enriched bone marrow cells (around 5% of the total bone marrow) isolated from the low density layers of a discontinuous Ficoll gradient. HDC levels are markedly enhanced after a 24 h incubation with IL-3 while a 4 h exposure results only in a slight activation. It results from increased expression of the mRNA coding for HDC, as assessed by Northern blot analysis after a 24 h incubation with IL-3. At the same time point and after a 4 h stimulation, we have evaluated the percentage of cells in this population which express HDC mRNA in response to IL-3, using *in situ* hybridization with the antisense riboprobe. We have thus established that enhanced HDC mRNA expression occurs in a small immature subset representing from 5 to 8% of the progenitor-enriched bone marrow cells.

In addition to its central role during immediate hypersensitivity, histamine has been implicated by several authors as a mediator of cell growth and/or differentiation. This notion originates from the report of Kahlson and Rosengren (1), showing that the regeneration of injured tissues is associated with increased histamine synthesis. In more recent years, several pharmacological studies dealing with the effect of histamine on hematopoiesis have contributed to strengthen this hypothesis. Indeed, exogeneous histamine has been shown to trigger both pluripotent stem cells (CFU-S) and colony-forming cells (CFC) (2,3) into cell cycle and to induce differentiation of myeloblasts into myelocytes and metamyelocytes (4). Furthermore, the demonstration of an intracellular

0006-291X/93 $4.00

histamine receptor and the inhibition of Con-A-induced lymphocyte proliferation by its antagonist have led to the assumption that histamine could act as a second messenger of cell proliferation via this new type of receptor (5).

Our own evidence, showing that large amounts of histamine are generated in the hematopoietic environment in response to IL-3, indicates that the aforementioned observations could be of physiological relevance. Indeed, we have characterized several years ago a new biological activity of IL-3, termed histamine-producing cell stimulating activity (HCSA), which consists in a striking induction of histamine production by bone marrow cells (6,7). We have also shown that this endogeneous histamine is requisite for the CFU-S cell cycling promoted by IL-3 (8). It is particularly important to stress that HCSA is quite distinct from the degranulation of histamine stored inside mast cells or basophils since it is entirely due to an active synthesis of the amine (9). Mast cells, their committed precursors and other mature components of the bone marrow are not involved in this biological activity (10). We therefore postulated that its expression might be an early event in IL-3-induced cell differentiation taking place in various hematopoietic progenitors (8). We also provided evidence that IL-3 can induce HCSA in vivo, either directly after injection, or indirectly after antigenic challenge of immunized mice which results in the appearance of endogeneous IL-3 (11).

The finding that actinomycin D and cycloheximide inhibit IL-3-induced histamine formation provided a first indication for the occurence of *de novo* synthesis of HDC in response to the growth factor (9). The aim of the present study was to examine more precisely the mechanism involved in our phenomenon by analysing the effect of IL-3 on the expression of the mRNA coding for HDC. We demonstrate herein that the enhanced intracellular HDC activity in response to the growth factor is associated with increased mRNA expression in hematopoietic cells, as established by Northern blot analysis and in situ hybridization.

MATERIAL AND METHODS

Preparation of progenitor-enriched bone marrow cells-Bone marrow cells from C57BL/6 mice (Charles River, St Aubin les Elbeuf, France) were removed by flushing of tibiae and femurs with ice-cold Hanks' balanced salt solution (HBSS; Gibco Europe, Paisley, Scotland). After centrifugation, they were suspended in serum-free minimum essential medium with Earle's salts (MEM; Gibco) supplemented with 1% L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate 100 X, 100 IU/ml penicillin and 100 µg/ml streptomycin (all from Gibco). Bone marrow cells were then fractionated on a discontinuous Ficoll gradient, as previously described (6). Briefly, the gradient was prepared from Ficoll 400 (Pharmacia Fine Chemicals, Uppsala, Sweden) at concentrations of 10.0, 14.6, 16.1, 17.7, 19.2 and 23% (w/w) in 0.1 M sodium phosphate (pH 7.4). Bone marrow cell suspensions (1-2 ml) from five mice were layered on top of the gradient (1.2 ml/layer) and centrifuged for 30 min at 23,500 g at 4°C. Layer 0 was defined as the interphase between culture medium and the less dense layer and subsequent interphases were numbered sequentially. Cells from layers 2 and 3 were collected, washed, and incubated at a final concentration of 10^6 cells/ml, with or without murine recombinant (mr) IL-3 (British Biotechnology, Oxon, UK) at a concentration of 1 ng/ml. These cells represent 3-5% of the unfractionated bone marrow and are enriched for CFC, CFU-S, and mast cell precursors (9). They are hereafter referred to as progenitor-enriched bone marrow cells (PEBMC).

Histamine and histidine decarboxylase assay - Histamine concentrations were assayed by an automated continuous flow fluorometric technique (12). HDC activity was measured by a radiochromatographic assay (13) comprising minor modifications. Briefly, cell pellets were resuspended in 50 mM ice-cold phosphate buffer (pH 7.4) and gently sonicated. Aliquots of the HDC-containing suspension were then incubated in 50 mM phosphate buffer at a final concentration of 10 µM pyridoxal 5'-phosphate and 0.1 µM L-[^{3}H] histidine (specific activity : 50 Ci/mmol). Incubations were always performed under conditions of initial velocity measurement and were stopped (generally after 120 min) by the addition of perchloric acid (0.4 N, final concentration) containing 0.3 M unlabeled histidine to minimize possible non-specific decarboxylation of remaining L-[^{3}H] histidine. The specificity of the reaction was assessed for each sample by performing the same determination in the presence of 10^{-5} M α-fluoromethylhistidine (a specific inhibitor of HDC) considered as blank value. After centrifugation, in order to eliminate proteins and precipitated $KClO_4$, the synthetized L-[^{3}H]-histamine was separated from [^{3}H]-histidine by ion exchange chromatography on Amberlite CG-50 columns.

Hybridization probes - The murine HDC probe used for Northern blot analysis and *in situ* hybridization was obtained as previously described (14). A 523 bp fragment was subcloned in PGEM-4 plasmed vector (Promega Biotec). The murine β-actin probe (a generous gift from F. Dautry, Villejuif, France), subcloned into a bluescript plasmid vector served as positive control.

Northern blot analysis - Total RNA were isolated from PEBMC by a guanidine isothiocyanate procedure (15), followed by cesium chloride centrifugation. Five µg of total RNA were electrophoresed in a 1% agarose-formaldehyde gel, transferred to a nylon membrane and baked for 2 h at 80°C. HDC and β-actin probes were labeled with [^{32}]P dCTP by random priming, according to the manufacturer's instructions (Megaprime DNA labeling system, Amersham, GB). Filters were prehybridized for 18 h in hybridization buffer (HBN : 50% deionized formamide, 5 X SSC (1 X SSC = 150 mM NaCl, 15 mM Na-citrate, pH 7), 5 X Denhartdt's, 0.1% SDS, 125 µg/ml DNA SS) at 42°C and then hybridized in the same conditions for further 18 h with the radiolabeled probe (10^6 cpm/ml). Filters were washed once for 30 min at room temperature with 1 X SSC, 0,1% SDS and twice at 68°C in 0.1 X SSC 0.1 SDS. Autoradiography was then performed during 72 h at -80°C. Rehybridization of the same filters with a β-actin probe was performed in order to check the amount of RNA loaded per lane.

In situ hybridization - The technique used was based on the work of Lawrence et al. (16) and Cox et al. (17), with some modifications. In brief, low density cells from the Ficoll gradient, previously incubated for 4 h with or without 1 ng/ml of mrIL-3, were cytocentrifuged and immediately fixed in 4% paraformaldehyde in PBS for 3 min at room temperature. Preparations were then transferred into a solution of 70% ethanol and stored at 4°C until use. Run-off transcripts of appropriate linearized plasmid were synthetized using either T7, T3 or SP6 RNA polymerase in a reaction medium containing 50 µCi [a^{35}S]-UTP (1200 Ci/mmol; Amersham, Les Ulis, France), according to the manufacturer's instructions (Promega Biotec), except that unlabeled UTP was omitted in order to synthetize RNA probes with a specific activity of 10^9 cpm/µg. Their fragment length was adjusted to a mass average of approximately 100-150 bases by limited alkaline hydrolysis. RNA probes were ethanol-precipitated with yeast tRNA and salmon sperm DNA and washed with 70% ethanol. The dried pellets were resuspended in the hybridization buffer to give final concentrations of 50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 8), 5.5 mM EDTA, 1 X Denhardt's, 10% dextran sulfate, 20 mM DTT, 250 µg/ml yeast tRNA, 12.5 µg/ml salmon sperm DNA, and 10^6 cpm/30µl of the RNA probe. The slides were post-fixed in 2% paraformaldehyde/1% glutaraldehyde in PBS for 5 min, washed in PBS, acetylated by 0.25% acetic anhydride in 0.1 M triethanolamine buffer (pH 8) for 10 min, washed in PBS, dehydrated in ethanol, and air dried. Negative controls were prepared by treating some slides with RNAse A (200 µg/ml) and RNAse T1 (12.5 U/ml) (Boehringer, Mannheim, RFA) in 2 X SSC. The hybridization mixture was denaturated, applied to slides and covered with parafilm. Hybridization was performed at 50°C for 18 h in a humidified chamber. The subsequent washing procedures were all carried out in the presence of 10 mM DTT at 52°C, when not otherwise specified. Slides were washed successively for 5 min in a) 2 X SSC, b) 50% formamide, 2 X SSC, c) 2 X SSC, followed by a RNAse A digestion (10 µg/ml in 2 X SSC) for 10 min at 37°C in the absence of DTT. The slides were then washed successively for 5 min in a)50% formamide, 2 X SSC for 5 min b) 2 X SSC, c) 1 X SSC,

d) 0.5 X SSC, e) 0.1 x SSC and dehydrated in ethanol. For autoradiography, the slides were dried and coated with NTB2 emulsion (Eastman Kodak). After 3 weeks of exposure, the slides were developed in Dektol (Eastman Kodak), fixed in Unifix (Eastman Kodak) and stained with May-Grünwald Giemsa.

RESULTS AND DISCUSSION

The hematopoietic growth factor IL-3 is a potent inducer of histamine production by cells from murine hematopoietic organs, i.e. bone marrow, fetal liver and to a lesser degree, spleen (18). This stimulatory effect, termed histamine-producing cell stimulating activity (HCSA) is particularly striking in bone marrow cells located in the low density layers of a discontinuous Ficoll gradient, as shown in fig. 1. The most active cell fraction represents on the average 3-5% of the total bone marrow and contains the bulk of hematopoietic progenitors, such as colony-forming cells (CFC), colony-forming units in spleen (CFU-S) and mast cell precursors (MCP)(9). This population, referred to as progenitor-enriched bone marrow cells (PEBMC), produces 5-10 times more histamine in response to IL-3 than unfractionated bone marrow and has therefore been currently used in this study. The involvement of mast cell degranulation in IL-3-induced histamine production by these cells can be ruled out for the following reasons: a) the very low histamine content at the onset of culture (less than 5 ng per 10^6 cells); b) the abrogation of the biological activity by 10^{-5} M a- fluoromethylhistidine (αFMH), a specific inhibitor of the histamine-forming enzyme (HDC); and c) the enhanced HDC activity in lysates from PEBMC stimulated by IL-3. As illustrated in fig. 1, this enhancement is weak but detectable after a 4 h incubation (2.1 ± 0.9 fold increase ; mean value ± SEM from 4 experiments) and increases over 24 h (12.5 ± 2.8 fold increase; mean value from 4 experiments). It is noteworthy that PEBMC contain a significant spontaneous HDC activity which persists after 24 h of culture in medium alone.

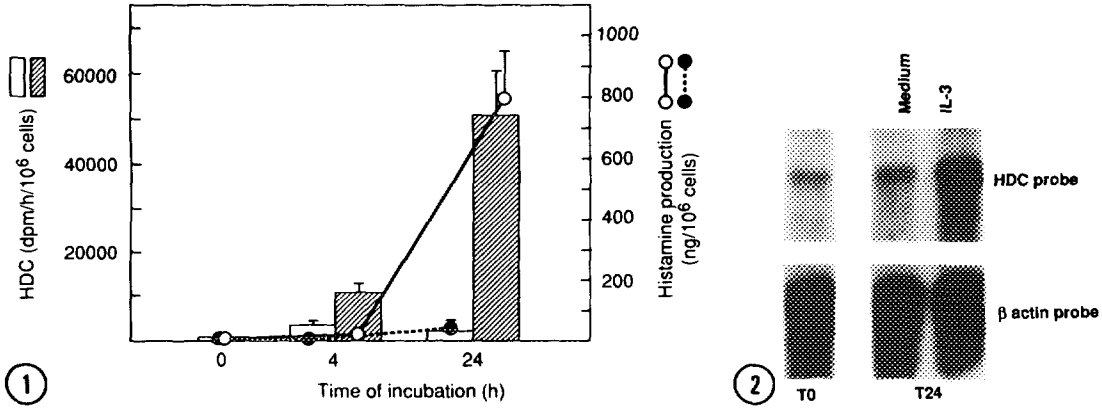

Fig. 1. Effect of IL-3 on histamine production (O—O with IL-3; ●----● without IL-3) and intracellular histidine decarboxylase activity (▨ with IL-3; ▭ without IL-3) in progenitor-enriched bone marrow cells.

Fig. 2 . Northern blot analysis: expression of histidine decarboxylase mRNA in progenitor-enriched bone marrow cells in response to IL-3.

The inhibition of IL-3-induced histamine synthesis by actinomycin D and cycloheximide (9) provided a first argument for the requirement of *de novo* synthesis of HDC in this biological activity. Taking advantage of the recent cloning of the HDC gene, we performed Northern blot analysis using total RNA from PEBMC and the HDC cDNA probe. As shown in fig. 2, the latter hybridized with a 2.7 kb RNA from PEBM cells which is in accordance with the size of the messenger. The hybridization signal is clearly increased after a 24 h incubation of PEBMC with IL-3, as compared with controls. This enhancement of HDC mRNA expression in response to IL-3 is not consistently observed at 4 h. In order to verify whether a certain percentage or the totality of cells express HDC mRNA, in situ hybridization experiments were performed on PEBM cells after 4 or 24 h of incubation in culture medium with or without IL-3 (fig. 3). The specificity of hybridization was assessed by the lack of labeling in the presence of the sense probe, as well as ribonuclease treatment of some cell preparations. Background signal was estimated to be less than 10 silver grains per cell. The integrity of the mRNA was verified by a positive control, performed with a β-actin probe. A 4 h incubation with IL-3 results in a significant increase in the percentage of PEBMC labeled with the HDC mRNA probe ($5.6 \pm 0.7\%$ versus $1.5 \pm 0.3\%$ in the population incubated with culture medium alone; means ± SEM from 6 experiments). For comparison, 0.9 ± 0.2 % of the cells are labeled spontaneously, before incubation. The growth factor enhances the number of positive cells and the intensity of labeling, suggesting that it exerts its action by increasing the expression in cells already synthetizing HDC and by inducing it in negative cells. After 24 h of incubation with IL-3, the % of labeled cells is only slighly increased (8.3%)but the labeling is more intense. These results are in agreement with the small increase in HDC activity in PEBMC lysates already observed within 4 h of incubation with IL-3, but not with the unconsistent increase in mRNA levels in PEBM cells assessed by Northern blot analysis at the same time point. This discrepancy might be explained by a lack of sensitivity of this technique due to the use of total mRNA for hybridization. Given the small number of PEBMC recovered from the low density layers of the discontinuous Ficoll gradient, we did indeed not have the opportunity to use poly (A) mRNA which would probably have facilitated such an early detection. This rapid increase in HDC mRNA expression supports the notion that IL-3-induced histamine synthesis does not result from the proliferation of HDC-containing cells. The lack of effect of irradiation or mitomycin C treatment on histamine production (9) proves that this is effectively the case. The morphology of the cells with increased HDC mRNA expression is consistent with our previous report showing that mast cells or their committed progenitors are not responsible for HCSA (10). Indeed, histamine-producing cells do not have the features of mature mast cells, the majority of the most heavily labeled cells being of blast-like appearence. On the other hand, the percentage of labeled cells is much higher than MCP frequencies in this population (0.3%), supporting the notion that HCSA is not restricted to the mast cell lineage but shared by a larger range of hematopoietic progenitors (10).

In conclusion, we clearly demonstrate that IL-3 increases HDC mRNA expression in hematopoietic progenitors. This finding gives further support to the notion that

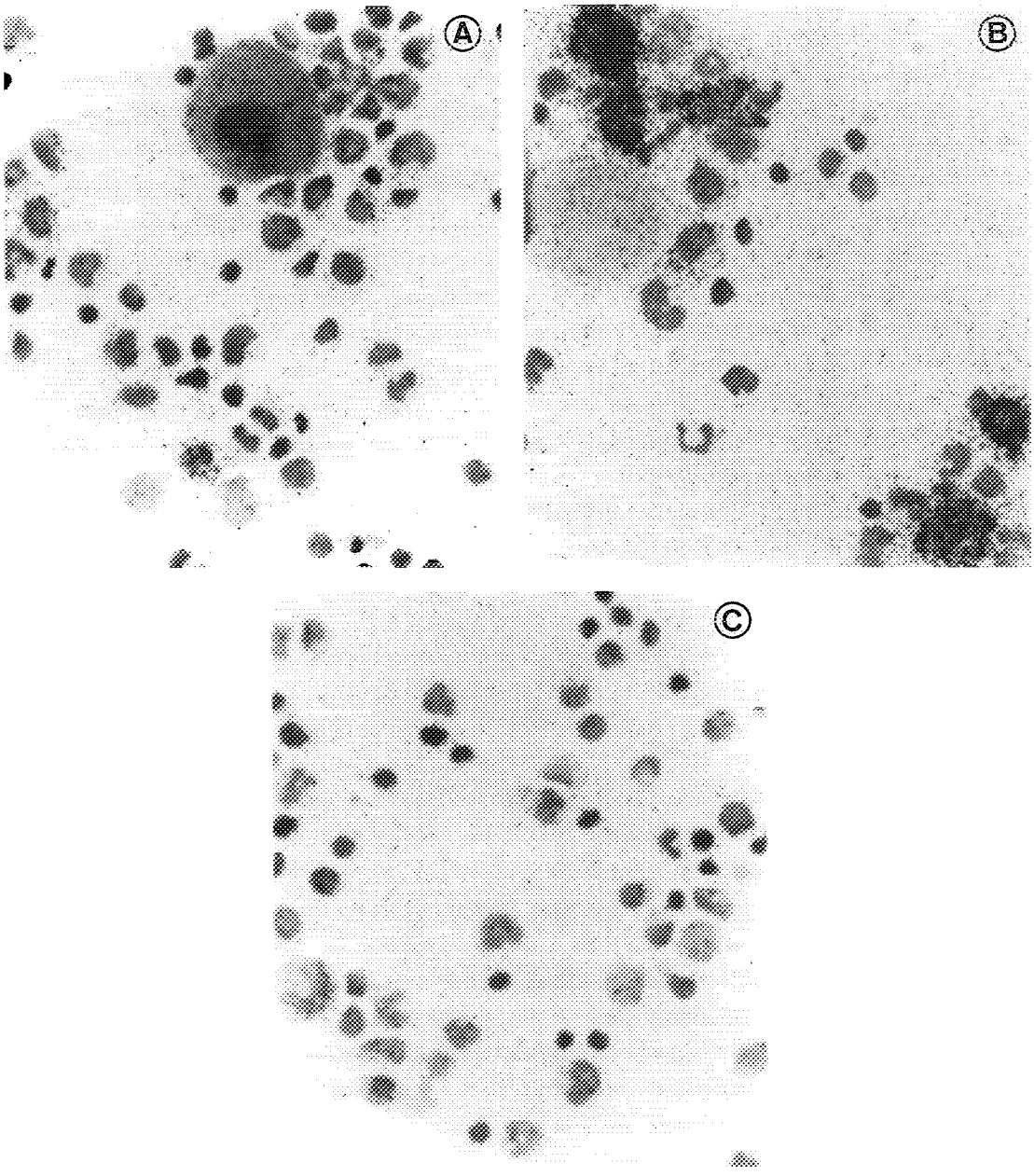

Fig. 3 . *In situ* hybridization. Low density cells were incubated for 24 h with culture medium (A) or 1 ng/ml of IL-3 (B). HDC transcripts were detected by an antisense HDC RNA probe, while the sense RNA probe served as a negative control (C). The latter gave the same results as ribonuclease pretreatment (data not shown). Exposure time was three weeks and magnification X300.

histamine may play a role during hematopoiesis particularly in response to IL-3, as already suggested by the finding that IL-3-induced CFU-S cycling does no longer occur if the synthesis of histamine in response to the growth factor or its binding to H_2 histamine receptors is inhibited (8). In addition, the potential role of histamine in the

control of cell proliferation has to be reconsidered in the light of the newly discovered intracellular histamine receptor located both in microsomes and in the nucleus (19) which has been shown to be involved in the control of platelet aggregation (20) and cell proliferation (21). Moreover, IL-3 induces both an increase in HDC and ornithine decarboxylase (ODC)(22). The fact that the latter is actually claimed to be a new protooncogen (23), together with the observation that both enzymes are coexpressed in response to the same stimulus make an evaluation of the control mechanisms they may exert in concert during the cell cycle extremely promising.

ACKNOWLEDGMENTS

The authors wish to thank A. Arnould for her excellent technical assistance. This work has been partially supported by the Association pour la Recherche contre le Cancer ARC N° 6365.

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