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IN VIVO AND *IN VITRO* INHIBITION OF HUMAN HISTIDINE DECARBOXYLASE BY (*S*)-α-FLUOROMETHYLHISTIDINE

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Abstract—Histidine decarboxylase (HDC) activity in Ficoll–Hypaque purified human peripheral blood leukocytes (PBL) was determined by measuring the formation of [^{3}H]histamine from L-[^{3}H]histidine. HDC activity was inhibited *in vitro* to more than 90% by (*S*)-α-fluoromethylhistidine (α-FMH) at concentrations of 10^{-5} M and above. Both polymorphonuclear and mononuclear cells possessed HDC activity, but on a per cell basis the former had several-fold higher enzyme activity than the latter. In safety and tolerability studies, α-FMH was administered orally to healthy human subjects twice daily for 7 days at doses of 2.5, 10, 50 and 100 mg per person. A dose-dependent inhibition of HDC activity was observed in PBL that were isolated both at 12 hr after administration of the first dose of α-FMH and after treatment for 1 week. At the 50 and 100 mg doses of α-FMH, there was complete inhibition of HDC activity and partial inhibition at the 10 mg dose. Twenty-four hours after the last dose, HDC activity had recovered to 64–100%, 44–46%, and 30–52% of control values in subjects that received 10, 50 and 100 mg α-FMH respectively.

Histamine is present in mammalian tissues in amounts ranging from less than 0.1 μg/g tissue or cells in blood to over 50 μg in gastric mucosa and lung and in excess of 1 mg in mastocytoma cells [1, 2]. Histamine plays a role in a number of physiological and pathological conditions, such as allergies (e.g. allergic rhinitis, asthma, urticaria and generalized anaphylaxis), inflammation, gastric acid secretion leading to peptic ulceration, neurotransmission, cardiac dysfunction, rapid tissue proliferation and possibly immunoregulation [3–14].

Histamine-mediated disorders are likely to be caused by an abnormal production, excessive release and/or enhanced tissue reactivity to histamine. At present, therapy is directed towards the prevention of immunoglobulin E-mediated histamine release or toward the blockage of the effects of histamine on receptors [6, 7]. Desensitization, an antigen-specific treatment, is not very effective, and histamine antagonists have a number of side-effects. Therefore, the ability to selectively inhibit histamine synthesis, thus lowering the histamine content in tissue mast cells and circulating basophils, could be a valuable alternative treatment.

Histidine decarboxylase (HDC; L-histidine decarboxylase, EC 4.1.1.22) is the sole enzyme involved in the formation of histamine, and this enzyme has been identified in extracts of many tissues [3, 15–21]. A number of compounds have been developed as inhibitors of *in vitro* HDC activity [22–28] but most of these were not as effective *in vivo*. Recently, Kollonitsch *et al.* [29, 30] reported the synthesis of a series of α-fluoromethyl derivatives of amino acids and demonstrated that these are irreversible inhibitors of the corresponding amino acid decarboxylases [31, 32]. One of these compounds, (*S*)-α-fluoromethylhistidine (α-FMH), is a specific inhibitor of histidine decarboxylase, and it has been shown in a number of animal species to be effective in inhibiting HDC activity and in reducing histamine level [33–37].

To demonstrate the inhibitory effect of α-FMH in human subjects, it was necessary to develop an assay for HDC using material that can be obtained conveniently. Enzyme activity could not be measured in extracts from peripheral blood cells but it could in whole cell preparations. In this paper we report on this assay and its applications in studies with human subjects who received α-FMH at different dose levels.

MATERIALS AND METHODS

Materials. Drugs and reagents were obtained from the following sources: Ficoll 400, Pharmacia Fine Chemicals, Piscataway, NJ; diatrizoate sodium (Hypaque), Sterling Drug Inc., New York, NY; phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS), *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes), neuramindase and Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS), Grand Island Biological Co., Grand Island, NY; L-histidine monohydrochloride monohydrate, pyridoxal phosphate and human serum albumin (HSA), Calbiochem-Behring, La Jolla, CA; Bio-Rex 70, Bio-Rad Laboratories, Richmond, CA; and (*S*)-α-fluoromethylhistidine, Dr. J. Kollonitsch, Merck Sharp & Dohme Research Laboratories. Radio-

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active materials were obtained from the Amersham Corp., Arlington Heights, IL. All solutions were prepared with analytical grade chemicals.

Purification of [^{3}H]histidine. L-[2,5-^{3}H)Histidine, specific activity 53 Ci/mmole, (1 mCi in 1 ml) was applied to a 3-ml packed volume of Bio-Rex 70 resin in a polystyrene column with plastic filter disc (Isolab Inc., Akron, OH) and eluted with 3 ml of Hepes-buffered HBSS containing 2% ethanol.

Isolation of peripheral blood leukocytes (PBL). All steps were carried out at room temperature. Venous blood was drawn into 30 or 50 ml syringes containing 10% EDTA solution, pH 7.2 (0.4 ml/10 ml blood), and 10 ml of this blood was diluted with 30 ml of sterile PBS in a 50-ml Falcon conical polystyrene tube and underlayered with 10 ml Ficoll–Hypaque, sp. g. 1.086. After centrifugation at 400 *g* for 30 min, PBL at the interface were collected, diluted with 40 ml of sterile PBS, and pelleted at 400 *g* for 10 min. The cell pellet was suspended gently in the assay buffer which consisted of 10 mM Hepes-buffered HBSS containing 0.1% HSA (HBSS–HSA). This procedure resulted in greater than 95% PBL viability, and the number of PBL recovered per ml of blood ranged from 1.6 to 2.5×10^6. Unless otherwise stated, the volume of cell suspension was adjusted with Hepes-buffered HBSS–HSA to a cell density of 10^7/ml.

Histidine decarboxylase assay. HDC activity in human PBL was measured in 17×100 mm Falcon round bottom tubes with 1 ml of cell suspension containing 10^7 cells, 2×10^{-7} M unlabeled L-histidine and 2.5 μCi purified [^{3}H]histidine (see above). The reaction was carried out at 37° in a water bath, and the contents of the tubes were mixed every 15–20 min. After 4 hr the reaction was terminated by the addition of 0.2 ml of a 3.4 M perchloric acid solution containing 0.47 M L-histidine. The contents were mixed and centrifuged at 1000 *g* for 10 min. The supernatant fraction from each tube was adjusted to pH 8.0 by the addition of 1.25 M KOH solution prepared in 0.05 M Tris–HCl buffer (pH 8.0), and applied to a 2-ml Bio-Rex 70 resin column. The resin was washed with 10 ml of 0.02 N HCl such that L-histidine was removed from the column whereas histamine, the product of enzyme reaction, remained bound to the resin. Histamine was eluted with 3 ml of 2 N acetic acid directly into a liquid scintillation glass vial. Aquasol (New England Nuclear, Boston, MA) (14 ml) was added, and the contents were mixed and counted in a Beckman LS 8000 liquid scintillation counter.

Generally, three different reaction mixtures were prepared in duplicate for every PBL preparation: (1) incubation of the complete reaction mixture for 4 hr at 37°; (2) incubation of the complete reaction mixture for 4 hr at 37° in the presence of 10^{-4} M α-FMH; and (3) incubation of the complete reaction mixture for 4 hr at 37° in the absence of PBL. HDC activity in the presence or absence of α-FMH was expressed as counts per minute (cpm) corrected for background cpm obtained from reaction No. 3 (100–200 cpm).

During these studies it was observed that minor contamination of [^{3}H]histidine with [^{3}H]histamine raises the background and reduces the sensitivity of the assay. Therefore, it was necessary to purify [^{3}H]histidine by ion-exchange chromatography prior to use (see above).

Fluorometric determination of histamine. A perchloric acid (PCA) extract from 3×10^7 cells containing 1 mg exogenously added histamine was applied to a 5-ml Bio-Rex 70 column, washed with 20 ml of 0.02 N HCl, and eluted with a 100-ml gradient of acetic acid (0–2 N). Twenty microliters of each fraction was mixed with 2 ml of OPA reagent (50 mg *o*-phthalaldehyde in 5 ml methanol was mixed with 95 ml potassium borate buffer and, after degassing, 0.2 ml β-mercaptoethanol was added). Fluorescence was determined at λ_{ex} 366 nm and λ_{em} 455 nm and expressed as relative fluorescence units (i.e. the percent fluorescence relative to that induced by 10^{-6} M quinine sulfate in 0.1 M H_2SO_4).

[^{3}H]Histidine uptake. Incubation conditions as well as those used for cell preparation were the same as described above. After a 4-hr incubation, cells were pelleted by centrifugation at 400 *g* for 10 min, and then washed once in HBSS–HSA; radioactivity was determined in the cell pellet and the combined supernatant fractions.

Fractionation of PBL into mononuclear cells and polymorphonuclear cells. Peripheral blood leukocytes isolated by the above procedure were fractionated further by Ficoll–Hypaque centrifugation. Five milliliters of cells (10^7/ml in RPMI 1640 medium with 10% fetal calf serum) were layered over 3 ml of Ficoll–Hypaque, sp. g. 1.077 in 17×100 mm Falcon round bottom tube. After centrifugation at 400 *g* for 30 min, mononuclear cells (lymphocytes and monocytes) were at the interface whereas polymorphonuclear cells had pelleted. Both cell preparations were washed with PBS, pelleted, suspended in HBSS–HSA buffer, and assayed for HDC activity along with unfractionated cells.

Subjects and study design. To determine the *in vivo* effect of α-FMH, twenty-two healthy male volunteers aged 18–36 years received orally every 12 hr in a single-blind, placebo-controlled fashion fourteen doses of either 2.5, 10, 50 or 100 mg α-FMH. The twenty-two subjects were divided into four panels, and four subjects in each panel received α-FMH at one of the four dosage levels and one or two received placebo. Blood was collected and PBL were isolated for HDC determination 12 hr before and 12 hr after each subject's first dose, and 1 hr after and 24 hr after his last dose. The study was approved by the Thomas Jefferson University Institutional Review Board, and informed consent was obtained from all volunteers. Subjects consumed no other drugs from 1 week before until the end of the study.

For *in vitro* studies, PBL were obtained from the blood of healthy subjects at the Merck Sharp & Dohme Research Laboratories.

RESULTS

Characterization of the HDC reaction product. HDC activity was determined by measuring the conversion of [^{3}H]histidine to [^{3}H]histamine in the presence of PBL over a 4-hr incubation period, a time during which the conversion proceeded at a linear rate. Radiolabeled material that bound to Bio-Rex 70 and eluted with 2 N acetic acid was considered to

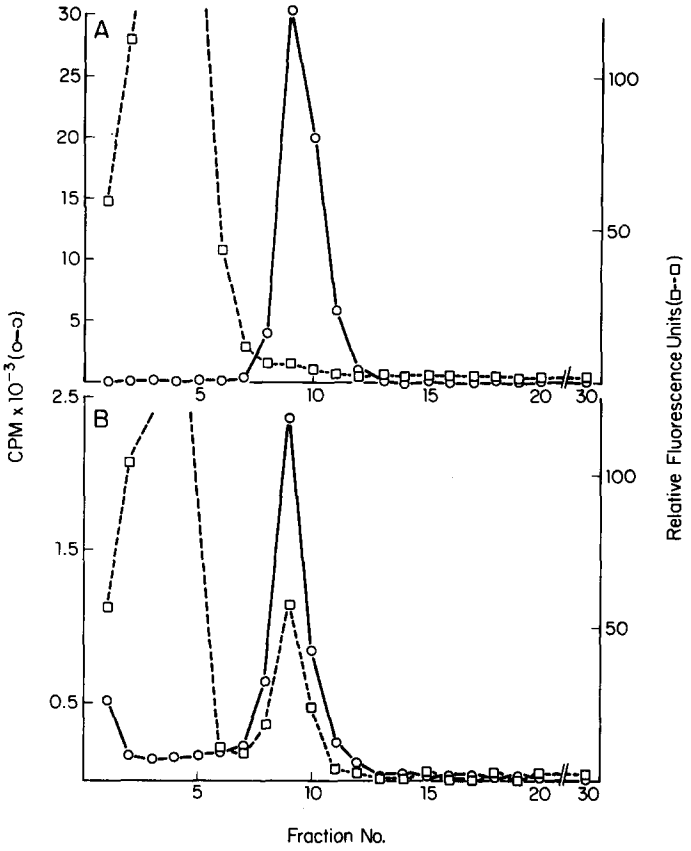

Fig. 1. Characterization of the HDC reaction product. (A) PBL (3×10^7) in 3 ml HBSS–HSA were extracted with PCA. [^{3}H]Histamine (0.5 μCi) was added, and the mixture was applied to a Bio-Rex 70 column. After washing with 20 ml HCL (0.02 N), the column was eluted with a 100 ml gradient of acetic acid (0–2 N). Fractions were assayed for radioactivity and for fluorescence. (B) PBL (3×10^7) in 3 ml HBSS–HSA were incubated for 4 hr with [^{3}H]histidine. A PCA extract was prepared and, after the addition of 1 mg unlabeled histamine, it was analyzed on a Bio-Rex 70 column as described in Fig. 1A.

be histamine. The following experiment was carried out to verify the nature of the reaction product. PBL were incubated with [^{3}H]histidine for 4 hr and extracted with perchloric acid. After the addition of unlabeled histamine, the extract was applied to a Bio-Rex 70 column and eluted with a gradient of acetic acid. Radiolabeled product eluted along with unlabeled histamine at an acetic acid concentration slightly less than 0.5 M (Fig. 1B). For comparison, a perchloric acid extract of PBL (not incubated with [^{3}H]histidine) was mixed with [^{3}H]histamine and fractionated on a similar column. Radiolabeled histamine eluted at the same acetic acid concentration as the enzyme reaction product (Fig. 1A).

HDC activity in human PBL and in vitro *inhibition by α-FMH.* Table 1 shows that the addition of α-FMH to PBL inhibited the formation of histidine.

Table 1. *In vitro* inhibition of HDC activity in peripheral blood leukocyte (PBL) by α-FMH*

Concn of α-FMH (M)	HDC activity† (cpm)	% Inhibition
10^{-5}	57	94
10^{-6}	284	72
10^{-7}	824	22
0	1020	0

* PBL (10^7) from a single human subject were assayed for 4 hr in the presence of α-FMH at the concentrations indicated.

† Counts per minute values have been corrected for the background counts (no incubation).

Table 2. [^{3}H]Histidine uptake by PBL in the presence of α-FMH*

α-FMH concn (M)	Incub. temp. (°)	Percent cell associated [^{3}H]histidine†
0	37	11.0
10^{-6}	37	9.4
10^{-5}	37	9.8
10^{-4}	37	10.4
0	0	2
10^{-4}	0	2.1

* Four-hour incubation.

† Percent cell associated =

$$\frac{\text{cpm in pelleted and washed cells}}{\text{cpm in pelleted cells + cpm in supernatants}}$$

Table 3. HDC activity in the presence or the absence of α-FMH in PBL isolated from normal human subjects

Subject No.	α-FMH (10^{-4} M)	[^{3}H]Histamine synthesized (cpm)	% inhibition by α-FMH
1	−	579	
	+	30	95
2	−	1491	
	+	59	96
3	−	1371	
	+	106	92
4	−	1901	
	+	21	99
5	−	664	
	+	34	95
6	−	708	
	+	101	86
7	−	1038	
	+	106	90
8	−	1237	
	+	0	100
9	−	698	
	+	90	87
10	−	2159	
	+	40	98

Table 4. HDC activity of PBL from normal human subjects

Subject	HDC activity on days* 0	1	2	3	7	8
1	707 (100)†	928 (131)	ND‡	ND	ND	ND
2	1155 (100)	1445 (125)	ND	ND	ND	ND
3	434 (100)	427 (98)	444 (102)	ND	ND	ND
4	1901 (100)	1705 (90)	2387 (125)	2340 (123)	ND	ND
5	2119 (100)	2630 (124)	ND	ND	2264 (107)	ND
6	1069 (100)	1297 (121)	ND	ND	1083 (101)	1089 (102)
7	1877 (100)	1795 (96)	ND	ND	1820 (97)	2142 (114)

* Expressed as cpm [^{3}H]histamine formed.
† Values in parentheses are percentages normalized to 100 day 0.
‡ ND = not done.

Near complete inhibition was observed at 10^{-5} M and over 70 and 20% inhibition at 10^{-6} and 10^{-7} M α-FMH respectively. The fact that α-FMH at a concentration as high as 10^{-4} M did not inhibit the uptake of [^{3}H]histidine by PBL (Table 2) suggests very strongly a direct inhibitory effect by α-FMH on the PBL-associated histidine decarboxylase.

To determine the range of HDC activity in PBL from different subjects, the enzyme activity was measured in PBL of ten normal adult healthy subjects (Table 3). PBL of each subject possessed measurable HDC activity though significant differences in enzyme activity were observed amongst individual subjects (579–2159 cmp of histamine synthesized). Results also show that the enzyme activity in PBL of each subject was inhibited strongly by α-FMH.

Changes in HDC activity in human subjects over a period of time. To assess the utility of this HDC assay as a tool to follow the effect of administration of α-FMH *in vivo* in human subjects, experiments were carried out to determine whether HDC activity in PBL obtained at different times from the same subjects remained constant. Table 4 shows that at different times HDC activity in PBL of a given subject varied by less than 30%. In two subjects, HDC activity was determined at intervals over a period of 6 months and found to be within the same range of reproducibility (results not shown).

HDC activity in fractionated PBL. PBL were fractionated in two separate experiments in a population enriched for mononuclear cells (lymphocytes and monocytes) and a population enriched for polymorphonuclear cells, and each was assayed for HDC activity. The results in Table 5 show that total HDC activity was divided about equally between both fractions, but that on a per cell basis HDC activity in the polymorphonuclear leukocyte fraction was four to five times higher than in the lymphocyte/monocyte fraction. Recovery of enzyme activity in the fractionated cells compared to total PBL was 100% in the first and 62% in the second experiment.

Table 5. HDC activity in fractionated PBL*

Expt. No.	Fraction	No. of cells	HDC/10^7 cells (cpm)	Total HDC (cpm)
I	Total PBL	4×10^7	1,262	5,048
	Mononuclear cells	3.3×10^7	861	2,841
	Polymorphonuclear cells	6.6×10^6	3,585	2,259
II	Total PBL	5.5×10^7	2,320	12,760
	Mononuclear cells	4.4×10^7	802	3,528
	Polymorphonuclear cells	1×10^7	4,375	4,375

* See Materials and Methods.

Table 6. *In vivo* inhibition of HDC activity in PBL of healthy subjects by α-FMH

Subject No.	α-FMH dose (mg)	HDC activity* on days†			
		0	1	7	8
1	2.5	1392	1463	1752	ND‡
2	P§	2119	2630	2264	ND
3	2.5	2263	2843	1923	ND
4	2.5	2055	2949	1234	ND
5	P	2150	3373	2316	ND
6	2.5	1995	2998	2034	ND
7	10	2242‖	798	664	1860
8	P	1078‖	1581	768	1399
9	10	1914‖	713	362	1296
10	10	1618‖	1033	786	1626
12	10	1406‖	634	203	1094
13	50	1945	157	224	853
14	P	1069	1297	1083	1089
15	50	1300	54	4	602
16	50	2118	285	41	946
17	P	1241	1659	2102	1860
18	50	1510	78	132	677
19	100	1521	0	58	460
20	P	1877	1795	1820	2142
21	100	2176	166	98	686
22	100	899	0	45	465
24	100	769	33	59	273

* Expressed as cpm [^{3}H]histamine formed.

† PBL for HDC assays were collected 12 hr prior to first dose of α-FMH (day 0), 12 hr after the first dose (day 1), after 7 days of twice daily dosing (day 7), and 24 hr after the last dose (day 8).

‡ ND = not determined.

§ P = placebo.

‖ Insufficient cells available on day 0 for HDC assay. Therefore, blood was collected 14 days after final dose of 10 mg α-FMH.

No further work was carried out to explain this difference in recovery.

HDC activity in human PBL after in vivo *administration of different concentrations of α-FMH.* Healthy male subjects were divided into four groups. One or two individuals per group received placebo, while the rest received α-FMH twice a day for 7 days at levels of 2.5, 10, 50 or 100 mg. HDC activity was determined in PBL obtained from blood collected at 12 hr before (day 0) and 12 hr after (day 1) each subject's first dose of α-FMH and 1 hr and 24 hr after the last dose (days 7 and 8 respectively). The results are shown in Table 6. HDC activity on days 1, 7, and 8 was also expressed as a percentage of the activity on day 0 which allowed for a direct comparison of the effect of each dose of α-FMH. These normalized values are shown in Fig. 2. All placebo subjects (except No. 20) showed an increase in HDC activity 12 hr after the first administration of α-FMH. This does not appear to be due to random day-to-day variation as shown in Table 4, since this would have resulted in decreases of HDC activity as well. HDC activity also increased in all four individuals 12 hr after receiving 12.5 mg α-FMH, but in two of these individuals it decreased to 85 and 60%, respectively, after 7 days. At a dose of 10 mg α-FMH HDC activity was inhibited from 35 to 63% after 12 hr and 52 to 86% after 7 days. Both 50 and 100 mg doses resulted in near complete inhibition of HDC activity.

Partial recovery of enzyme activity was observed at 24 hr after termination of α-FMH treatment. Recoveries to 68, 78, 83 and 100% of the original enzyme activities were observed in the four individuals who received 10 mg, and to 50% or less in individuals who had received 50 and 100 mg α-FMH.

In vitro *recovery of HDC activity.* The previous experiment (Table 6 and Fig. 2) was expanded to determine whether recovery of HDC activity was associated with the original cell population or was dependent on the recruitment into the blood of fresh HDC containing cells. PBL were obtained on day 7 from one placebo (No. 20) and three subjects (No. 19, 21 and 22) who had received 100 mg α-FMH, and they were incubated for 18 hr at a density of 2×10^6 cells/ml in RPMI 1640 medium containing 10% fetal calf serum at 37° in an atmosphere of 5% CO_2 in air. HDC activity was then determined and expressed as a percentage of the enzyme activity on day 0 and compared with HDC activity in PBL immediately after their isolation from blood at days 7 and 8. Figure 3 shows again that HDC activity in those subjects who received α-FMH for 7 days was reduced to less than 5%, and that the *in vivo* recovery in subjects 19, 21 and 22 after 24 hr was to 30, 32, and 52% of the original activity respectively. However, recovery of HDC activity after maintaining their PBL in culture for 18 hr was approxi-

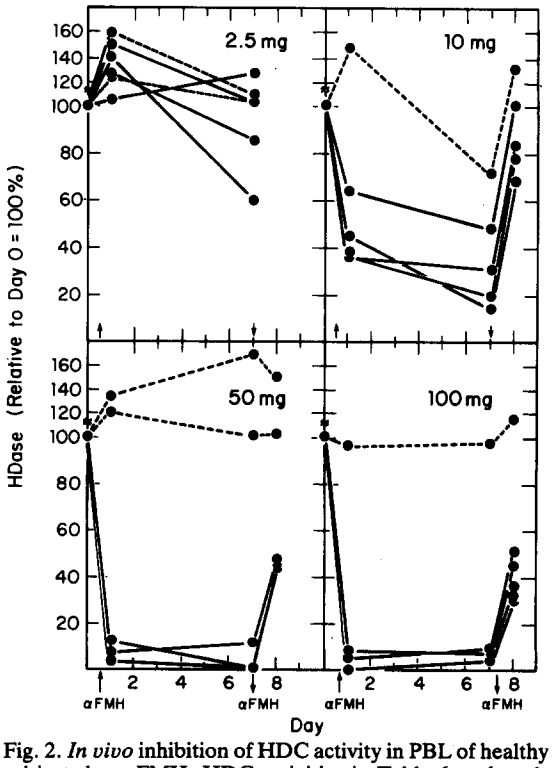

Fig. 2. *In vivo* inhibition of HDC activity in PBL of healthy subjects by α-FMH. HDC activities in Table 6 at days 1, 7, and 8 were expressed for each individual as a percentage of HDC activity at day 0. Key: (●——●) α-FMH treated; and (● - - - ●) placebo.

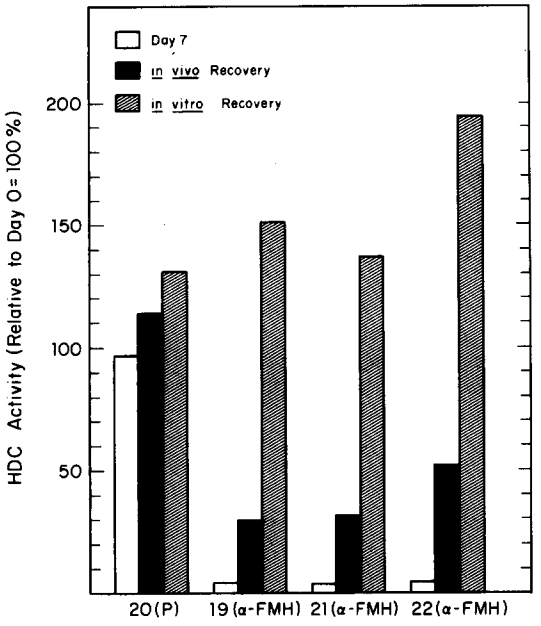

Fig. 3. Recovery of HDC activity after termination of the administration of α-FMH. Subjects 19, 21, and 22 received 100 mg α-FMH twice daily for 7 days; subject 20 was placebo. At 1 and 24 hr after the last dose of α-FMH, PBL were obtained and HDC activity was determined. Enzyme activity was expressed as a percentage of that on day 0 (see Fig. 2 and Table 6). Key (□) HDC activity in PBL collected 1 hr after last dose of α-FMH; (■) 24 hr after last dose of α-FMH. Part of the PBL collected 1 hr after the last dose of α-FMH were incubated for 18 hr at 37° and then assayed for HDC activity. Again HDC activity is expressed as a percentage of that on day 0 (▨).

mately 4-fold higher: 152, 137, and 195% respectively. HDC activity in PBL of the placebo control (subject 20) was 97 and 114% when collected on days 7 and 8 respectively. When control PBL collected at day 7 were maintained in culture for 18 hr, HDC activity increased to 131%.

RESULTS

The results presented in this paper show that: (a) HDC activity was measured in human PBL by following the conversion of [^{3}H]histidine to [^{3}H] histamine, (b) on a per cell basis polymorphonuclear leukocytes possessed several-fold higher enzyme activity than mononuclear cells, and (c) α-FMH was a potent and specific inhibitor of HDC activity in human PBL both *in vivo* and *in vitro*.

PBL isolated from all subjects tested possessed easily measurable HDC activity, with a 3-fold variation in enzyme level among individual subjects (Table 3). However, enzyme activity remained relatively constant over an 8-day period (Table 4). These results coupled with the ease of obtaining PBL for experimental purposes made it possible to utilize this HDC assay for exploring the efficacy of α-FMH to inhibit HDC activity *in vivo* in human subjects.

Oral administration of 50 or 100 mg α-FMH reduced HDC activity to less than 10% at 12 hr after the first dose, and the enzyme activity remained inhibited during the 7 days when subjects received these doses twice daily. Partial inhibition of HDC activity was observed after 12 hr and 7 days in subjects who received 10 mg, whereas inhibition was not observed 12 hr after administration of a single 2.5 mg dose, and partial inhibition (less than 40% in two subjects) after treatment for 1 week. HDC activity had recovered partially in PBL that were obtained 24 hr after termination of α-FMH treatment. The average recovery for each group (expressed as relative HDC activity on day 8 minus relative HDC activity on day 7; see Fig. 2) was 54, 41, and 33% for subjects who received 10, 50 and 100 mg α-FMH respectively. *In vitro* recovery of enzyme activity was determined by placing cells that were obtained 1 hr after the last dose of α-FMH in culture for 18 hr. Under those conditions, HDC activity recovered completely. Since inhibition of HDC by α-FMH is irreversible [29, 31, 32], enzyme recovery must be due to *de novo* protein synthesis.

These results extend published reports by others [33–37] who have shown for a number of animal species that α-FMH is an irreversible inhibitor of HDC activity in the brain, stomach and skin, and that chronic treatment with α-FMH lowers tissue histamine levels.

The fact that polymorphonuclear leukocytes are the main source of HDC activity in human PBL suggests that α-FMH inhibits histamine biosynthesis in circulating basophils. These cells along with tissue mast cells are mostly involved in histamine release during allergic reactions, and it is reasonable to expect that chronic treatment with α-FMH will deplete histamine levels and, thus, prove to be of therapeutic value in the treatment of allergic disorders.

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