HISTAMINE PRODUCTION DURING THE ANTI-ALLOGRAFT RESPONSE

Demonstration of a New Lymphokine Enhancing Histamine Synthesis*

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The role of histamine in immediate hypersensitivity has been recognized since the discovery of histamine. The in vitro release of histamine has been well correlated with in vivo allergic reactions (1, 2). Recent studies suggest that histamine may also play an important role in delayed hypersensitivity.

In the allograft situation, an increase in urinary histamine excretion has been found at the time of rejection (3). An increase in intracellular histamine formation by histidine decarboxylase activity has been demonstrated in rat skin allografts when compared with that obtained in rat skin autografts (4). The possibility that a histamine release plays a role in graft rejection has been suggested because inhibition of histidine decarboxylase activity induces prolonged allograft survival (5).

In addition to the effect of histamine in regulating microcirculation and in immediate hypersensitivity reactions, histamine may also modify leukocyte reactivity. Histamine may suppress or diminish the following: IgE-mediated release of histamine from mast cells and basophils (6), chemotaxis of eosinophils (7) and basophils (8), release of lysosomal enzymes from neutrophils (9), production or release of antibodies from lymphocytes (10), cytotoxicity of effector T cells (11), production of migration inhibitory factor (12, 13), antigen-stimulated cell proliferation (12, 13), and mitogen-stimulated cell proliferation (14).

Our work was carried out to study histamine production during an in vitro allogeneic response using the model of mixed leukocyte culture between donor and recipient of a mouse skin allograft. We report herein the discovery of a new lymphokine that is produced during the allogeneic reaction and that can induce a large increase in histamine synthesis. This factor was termed histamine-producing cell stimulating factor (HCSF).[1]

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[1] *Abbreviations used in this paper:* MLC, mixed leukocyte cultures; HCSF, histamine-producing cell stimulating factor; HBSS, Hanks' balanced salt solution; HDC, histidine decarboxylase; MHC, major histocompatibility complex.

Materials and Methods

Animals and Skin Grafting. C57BL/6 (H-2^b) and DBA/2 (H-2^d) and A/J (H-2^a) mice were purchased from Centre de Sélection et d'Elevage des Animaux de Laboratoire du Centre National de la Recherche Scientifique, Orléans La Source, France (C.S.E.A.L.). The following congenic mice were also used: B10 (H-2^b), B10D2 (H-2^d), B10BR (H-2^k), B10S (H-2^s), B10M (H-2^f), B10A (H-2^a), B10A5R, B10A2R, B10HTT, all obtained from C.S.E.A.L. B10AQR were obtained from Olac Ltd, Bicester, England.

Skin allografts were performed according to the technique described by Billingham and Medawar (15).

Mixed Lymphocyte Cultures. Animals were anesthetized with ether and killed by exsanguination. Spleens were removed, and cell suspensions were prepared by disruption with forceps. After washings with Hanks' balanced salt solution (HBSS), spleen cells were counted and adjusted to a final concentration of 10×10^6 viable cells/ml in minimum essential medium with Earle's salts (Flow Laboratories, Inc., Rockville, Md.) supplemented with 1% sodium pyruvate 100 X (Flow Laboratories, Inc.), 1% nonessential amino acid solution, 100 X (Flow Laboratories, Inc.), 1% L-glutamine (Flow Laboratories, Inc.), 100 IU/ml penicillin, and 100 μg/ml streptomycin. No serum was added. 1 vol of immunizing cell suspension plus 1 vol of recipient cell suspension were distributed in Falcon culture flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). 2 vol of each cell suspension were cultured alone as control. After 24, 48, or 72 h of incubation at 37°C in 5% CO_2, cell suspensions were centrifuged and cell-free supernates were removed and stored at −20°C.

Histamine Determination. Deproteinization of supernates was performed with perchloric acid (final concentration 0.4 N). Histamine content was then measured with the fluorometric method of Shore et al. (16) using an automated continuous flow technique. 60 samples of 100 μl each were treated per hour. With this technique, a highly reproductible linear relationship is obtained from 0 to 5 μg/ml of histamine base. Histidine may also influence the reaction but to a negligible degree because the molar fluorescence ratio (molar fluorescence of histamine: molar fluorescence of histidine) is 15,500. The reproductibility is good ($\leq \pm$ 5% for concentrations <2 ng/ml and from ±0.5 to 2% for greater concentrations). With this method, as little as 100 pg/ml of histamine can be determined.

Preparation of HCSF-containing Supernate. To eliminate histamine synthetized during the mixed lymphocyte culture (MLC), supernates of secondary MLC (and control supernates) were dialyzed for at least 24 h against distilled water, lyophilized, resuspended in culture medium (as described above), and then filtered on a 0.2-μm Nuclepore filter (Nuclepore, Corp., Pleasanton, Calif.). Such supernates were kept at −20°C before use.

Preparation of Cells. Spleen cells were prepared as described above for MLC. Lymph node cells were collected from mesenteric, inguinal, and axillary lymph nodes and disrupted in a Potter-Elvehjem homogenizer. Thymus cells were obtained by gently teasing the thymus with forceps. Peritoneal cells were obtained by washing the peritoneal cavity of normal C57BL/6 mice with HBSS that contained 10 IU/ml heparin (Liquemine Roche, Paris). Bone marrow cells were removed from the femurs of mice. Blood cells were obtained after Ficoll-Hypaque isolation. Immediately after collection, cells were washed twice with HBSS and resuspended in culture medium as described above. The final concentration of cells was 20×10^6 cells/ml for spleen, lymph node, and thymus; 20×10^6 or 6×10^6 cells/ml for bone marrow cells; 5×10^6 cells/ml for peritoneal cells; and 1×10^6 cells/ml for blood cells.

Assessment of HCSF Activity. HCSF-containing supernates were tested for their ability to induce histamine production from spleen or bone marrow cells by incubating 1 ml of cell suspension (i.e., 20×10^6 cells) with 1 ml of these supernates for 6, 24, 48, or 72 h at 37°C in a 5% CO_2 atmosphere. At the end of the incubation, cell suspensions were centrifuged, and cell-free supernates removed and stored at −20°C until histamine determination.

Monoclonal Anti-Mouse-Thy 1.2 Antibody. Monoclonal anti-mouse Thy 1.2 antibody was obtained from Becton Dickinson & Co., Mountain View, Calif. 10 ml of spleen cells (10×10^6 cells/ml) were incubated for 30 min at 4°C with 50 μg of purified monoclonal antibody in 50 μl of phosphate-buffered saline that contained 0.1% sodium azide. Mouse-absorbed guinea pig complement was then added. After 1 h of incubation at 37°C, cells were washed twice with HBSS. Control treatment was performed by replacing monoclonal antibody by 50 μl of

phosphate-buffered saline that contained 0.1% sodium azide. The trypan blue exclusion test performed after monoclonal antibody treatment showed an increase in mortality from 20 to 30% when compared with control treatment.

Mitomycin Treatment. Cells (10×10^6 cells/ml) were incubated for 40 min at 37°C with 50 μg/ml of mitomycin (Ametycine 2; Laboratoire Choay, Paris) and then washed three times. The effect of mitomycin was controlled by measuring [^{3}H]thymidine incorporation. For this purpose, aliquots of cell suspensions were cultured in flat-bottomed microplates (Microtest II; Falcon Labware, Div. of Becton, Dickinson & Co.) containing 10^6 cells/well. 18 h before the end of the incubation they were pulsed with 1 μCi [^{3}H]thymidine (Commissariat à l'Energie Atomique, Saclay, France). Each determination was performed on six samples. Cells were then harvested by an automated sample harvester, and the amount of [^{3}H]thymidine incorporation was evaluated by liquid scintillation counting.

Determination of Histamine Content in Cell Suspensions. After washings, cells were resuspended in distilled water. The total cellular content of histamine was then determined after disruption of the cells by heating aliquots of cells to 100°C for 10 min, or by freezing and thawing them three times.

Determination of Histamine Synthesis

TRANSFORMATION OF LABELED HISTIDINE INTO HISTAMINE. At the start of the cell culture, 72 nmol (4 μCi) of L-[2-^{14}C]histidine (Radiochemical Centre, Amersham, England) in 20 μl of an aqueous solution that contained 2% ethanol was added to 20×10^6 bone marrow cells suspended in 2 ml of control supernate (diluted 1:2) or 2 ml of HCSF-containing supernate (diluted 1:2). The final concentration of total histidine (labeled and unlabeled) was 0.20 mmol/liter. After 48 h of incubation with isotope at 37°C, cell suspensions were centrifuged. The cell pellets were solubilized with Soluene (Packard Instrument Co., Inc., Downers Grove, Ill.) and radioactivity was measured. Supernates were lyophilized and resuspended in 0.7 ml of distilled water. Deproteinization was then performed by adding 50 mg/ml of crystallized sulfosalicylic acid. After centrifugation, 500 μl of the supernate was applied to the column of a Technicon TSM1 analyzer (Technicon Corp., Tarrytown, N. Y.). The elution of the Technicon Chromobeads C3 column (23.5 × 0.5 cm) was performed at 57.5°C with a flow rate of 0.55 ml/min. Three sodium citrate-citric acid buffer solutions (0.1 mol/liter) were then used: (*a*) for 1 min, a pH 3.25 buffer solution that contained 6% (vol:vol) ethylene glycol monomethyl ester (Merck, Darmstadt, Federal Republic of Germany) and 0.5% (vol:vol) 2.2-thiodiethanol, (*b*) for 1 min, a pH 4.25 buffer solution, and (*c*) for 70 min, a pH 6.0 buffer solution containing 0.55 mol/liter NaCl. The elution was then performed by NaOH 0.4 mol/liter for 70 min. The effluent was separated into fractions of 1.1 ml. Each fraction was then mixed with 10 ml of dimilume (Packard Instrument Co., Inc.) and radioactivity was measured in a liquid scintillation spectrometer (Searle Delta 300; Searle Diagnostics Inc., Subsid. of G. D. Searle & Co., Des Plaines, Ill.).

10 μl of the initial solution of labeled histidine were also applied to the chromatographic column and results were corrected by substraction of the values obtained in this control chromatography.

DETERMINATION OF HISTIDINE DECARBOXYLASE ACTIVITY (HDC). Crude HDC extracts were obtained by freezing and thawing three times 20×10^6 cells suspended in 1 ml of distilled water. The homogenates were centrifuged at 3,000 *g* for 10 min at +4°C, and the supernates were assayed for HDC activity according to the method of Ritchie and Levy (17) with the following, slight modifications: No labeled histidine was used, and the production of histamine was measured using an automated spectrofluorometric technique. In brief, each tube contained the following reagents: 20 μl 250 mM phosphate buffer (pH 6.6 or pH 7.4), 10 μl pyridoxal-5′-phosphate (10^{-4} M), 10 μl L-histidine (10^{-2} M), 10 μl distilled water, and 50 μl crude histidine decarboxylase (HDC) extract (equivalent to 10^6 cells). These reagents were combined at +4°C in polystyrene tubes and mixed for 10 sec with a Vortex mixer (Vortex-Genie, Winn B. V., Tolbert, Holland). Reaction tubes were then incubated in a water bath at 37°C for 30, 60, 90, 120, or 180 min and shaken for 10 sec every 15 min. Histamine contents of crude HDC extracts and reagent blanks were carried through the entire procedure. 900 μl of 0.4 N perchloric acid was added immediately before incubation at 37°C to blank tubes (B) and at the end of the incubation tube to the reaction tubes (R) and other controls to stop the reaction.

Histamine was measured in the perchloric supernate. Each sample was assayed in triplicate and the resulting histamine content was averaged. Enzyme activity was calculated by substracting histamine content of (B) from that of (R) and expressed as picomoles of histamine formed/hour per 10^6 cells.

Characterization of HCSF

HEAT STABILITY. Aliquots of HCSF-containing supernate and control supernate were placed at room temperature or in water baths maintained at 56°C or 80°C for 10–30 min and tested thereafter for the action of HCSF.

ULTRAFILTRATION PROCEDURES. Supernates were ultrafiltered through the following Amicon membranes: XM 300, XM 100, XM 50 with respective molecular weight cut off of 300,000, 100,000, and 50,000 (Amicon Corp., Scientific Sys. Div., Lexington, Mass.).

ENZYME SENSITIVITY. HCSF-containing supernates were incubated for 3 h at 37°C with either 1 mg/ml of trypsin-30-enzygel, 1 mg/ml of chymotrypsin-enzygel, or 1 mg/ml of ribonuclease-enzygel. All these fixed enzymes were purchased from Boehringer, Mannheim, Federal Republic of Germany.

All treated fractions and controls were subsequently tested for their HCSF activity on bone marrow cells.

Discontinuous Density-Gradient Separation. A Ficoll gradient was prepared according to the method of Tak Yan Yu et al. (18), modified by Papiernik and Bach (19), and consisted of Ficoll 400 (Pharmacia Fine Chemicals, Uppsala, Sweden) at a concentration of 10% (wt:wt), 14.6%; 16.1%; 17.7%; 19.2% and 23% in 0.1 M sodium phosphate buffer, pH 7.4. The density of the gradient was controlled by measuring the refractive index with a Zeiss-Abbe refractometer (Carl Zeiss, Inc., New York). Refractive index of each layer (1.2 ml/layer) from the top of the gradient (10%) was: 1.352, 1.360, 1.363, 1.365, 1.367, and 1.374. Bone marrow cells were isolated as described above and suspended in culture medium. Bone marrow cells from 10 to 20 mice were layered on the top of the Ficoll gradient in 2 ml of culture medium. The gradient was then centrifuged for 30 min in an RC2 Sorvall centrifuge (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) at 23,500 *g* at 4°C. Layer 0 was defined as the interphase between the culture medium and 10% Ficoll; layer 1 was defined as between 10 and 14.6% Ficoll with subsequent interphases numbered sequentially. Cells from each of the six layers (the last one is the pellet) were washed in HBSS, counted, and adjusted to the final concentration of 0.5×10^6 cells/ml in culture medium. 60–80% of the cells were recovered.

Histochemical Procedures. Bone marrow smears were made, air dried, and stained with May-Grünwald Giemsa. For intracellular histamine detection, cell smears were treated with 1% ortho-phtaldialdehyde as described by Guy-Grand et al. (20). Alcian blue and toluidine blue stainings were performed as described by Miller and Walshaw (21).

Results

Histamine Production during MLC. As shown in Table I, a small amount of histamine is found in lymphocyte culture supernates. A slight increase in histamine production may be observed in a primary MLC after 24 h of culture (mean values of 5 experiments: 22.8 ± 16% of increase) and 48 h (mean values of 12 experiments: 34.6 ± 9.1%). A greater and more consistent increase is observed after 3 d of culture (mean values of four experiments: 86 ± 25.33%). The production of histamine is increased during a secondary MLC (allograft donor versus recipient). This increased production of histamine during the secondary MLC is detectable only after 24 h of culture (mean values of 5 experiments: 164 ± 53.5%), reaches its maximum at 48 h (mean values of 12 experiments: 259.75 ± 33.4%), and plateaus (mean values of 4 experiments: 208.5 ± 57.5%). At each corresponding period of incubation the increase in histamine production is significantly greater in secondary MLC than in primary MLC (Student's *t* test $0.01 \geqslant P \geqslant 0.001$).

The increase in histamine production during MLC performed between allograft

TABLE I

Histamine Production during Primary and Secondary MLC between C57BL/6 and DBA/2 Spleen Cells

		Histamine content after incubation of		
		24 h (5)*	48 h (12)	72 h (4)
			ng/ml	
Primary MLC (without previous allograft)	Control supernate	23.3 ± 2.8	22.8 ± 1.04	28.45 ± 3
	MLC supernate	26.10 ± 4.8‡	31.9 ± 3.3§	52.25 ± 7.9‖
Secondary MLC (performed 12 d after allograft)	Control supernate	28.9 ± 2.7	33.5 ± 2.3	38.2 ± 3.3
	MLC supernate	73.6 ± 12.8¶	123.8 ± 16.9**	125.9 ± 39.1**

* Number of experiments is in parenthesis.
‡ *P* compared with control: not significant.
§ *P* compared with control: <0.02.
‖ *P* compared with control: <0.05.
¶ *P* compared with control: <0.01.
** *P* compared with control: <0.001.

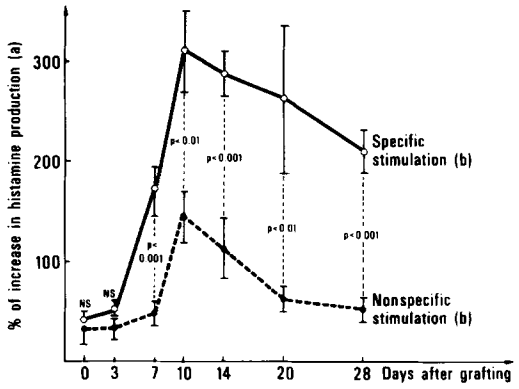

FIG. 1. Increased histamine production during secondary MLC performed at different intervals after grafting. Each point represents the mean values of nine mice. (a) Histamine production is expressed by the following formula:

$$\frac{\text{histamine production during MLC} - \text{histamine production during control culture}}{\text{histamine production during control culture}} \times 100.$$

(b) Specific stimulation: spleen cells from C57BL/6 mice grafted with DBA/2 skin cultured with DBA/2 cells. Nonspecific stimulation: spleen cells from C57BL/6 mice grafted with DBA/2 skin cultured with CBA cells.

donor and recipient appears 7 d after the graft, reaches its maximum during allograft rejection (day 10), remains high until day 20, and then begin to decrease (Fig. 1). Stimulation of recipient cells with third-party cells also induces an increase in histamine production, but to a lesser degree. The difference in histamine production between specific and nonspecific stimulation is significant (Student's t test $0.01 \leq P \leq 0.001$) (Fig. 1).

(a) Effect of Monoclonal Anti-Mouse Thy 1.2 Antibody Treatment. When spleen cells from allograft donor and recipient were pretreated by monoclonal anti-mouse Thy

1.2 antibody, the production of histamine during MLC is considerably diminished when compared with that of MLC performed with control cells that have been treated with mouse-absorbed guinea pig complement but without anti-mouse Thy 1.2 antibody; 71.3% of decrease in histamine production; $n = 6$: see Table II.

This suggests an involvement of T lymphocytes in the phenomenon.

(b) Effect of Mitomycin Treatment on Histamine Production during Secondary MLC. As shown in Table III, mitomycin treatment of donor cells does not modify histamine production during secondary MLC. Surprisingly, mitomycin treatment of recipient cells or of recipient and immunizing cells does not abolish histamine production although [^{3}H]thymidine incorporation is completely abolished. However, such a treatment induces a significant ($P < 0.05$) decrease in histamine production.

(c) Increase in Histamine Production during Secondary MLC Does Not Result from an Increase in Histamine Secretion. That the increase in histamine production during secondary MLC does not result from an increase in histamine secretion can be affirmed by determining histamine content in supernates and cells before and after the MLC. Results reported in Table IV show that a very small amount of histamine is present in cells before culture. After a 48-h incubation, the total histamine content (supernate and cells) is significantly higher in secondary MLC than in controls (C57BL/6 cultured alone plus DBA/2 cultured alone).

(d) Studies on the Specificity of the Stimulating Cell Inducing the Increase in Histamine Production During Secondary MLC. To investigate alloantigens coded by the major histocompatibility complex (H-2) necessary for the induction of the increase in histamine production during secondary MLC, we used congenic mice.

(i) Histamine Production During Secondary MLC Performed with Third-Party Cells Differing

TABLE II

Histamine Production during MLC after Monoclonal Anti-Thy 1.2 Antibody Treatment

MLC performed with	Percentage of increase in histamine production					
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6
Control-treated cells*	338	125	283	111	215	177
Anti-Thy 1.2-antibody-treated cells‡	84	30	92	20	63	61

* Control-treated cells: cells incubated with mouse-absorbed guinea pig serum without anti-Thy 1.2 antibody.

‡ Anti-Thy 1.2 antibody-treated cells: cells incubated with anti-Thy 1.2 antibody and mouse-absorbed guinea pig serum.

TABLE III

Effect of Mitomycin Treatment on Histamine Production during Secondary MLC

	Mitomycin treatment of	Percentage of increase in histamine production	P	[^{3}H]Thymidine incorporation
				cpm
48-h secondary MLC between skin allograft donor and recipient mice	No cells	280.6 ± 57.6		21,427 ± 3,331
	Recipient and immunizing cells	121.8 ± 35.8	<0.05	1,212 ± 321
	Immunizing cells	292.2 ± 64.8	NS*	16,095 ± 593
	Recipient cells	132.6 ± 35.7	<0.1	14,593 ± 2,239

* NS, not significant.

TABLE IV

Histamine Content in Supernate and Cells of Secondary MLC (a Typical Experiment)

	Histamine content		
	Cells	Supernates	Total
		ng	
Before culture			
20×10^6 spleen cells from DBA/2-grafted C57BL/6 mouse (in 2 ml)	6.8	4.4	11.2
20×10^6 spleen cells from normal DBA/2 (in 2 ml)	6.2	3.2	9.4
After 48 h of culture of control (cells cultured alone)			
20×10^6 spleen cells from DBA/2-grafted C57BL/6 mouse (in 2 ml)	6.6	65.2	71.8
20×10^6 spleen cells from normal DBA/2 (in 2 ml)	3.2	23.6	26.8
MLC			
20×10^6 spleen cells from DBA/2-grafted C57BL/6 mouse + 20×10^6 spleen cells from normal DBA/2 (in 4 ml)	25.6	460.2	485.7

at the Whole H-2 Region with Immunizing Cells. Maximum increase in histamine production is always found when recipient cells were cultured with immunizing strain cells. If, in each experiment, the percentage of increase in histamine production with immunizing cells is taken as 100% (after substraction of the percentage of increase during primary MLC with same cells), stimulation of spleen cells from allograft recipient with third-party cells differing at the whole H-2 region with immunizing cells induces between 3 and 23% of the response obtained with specific stimulation (Table V).

(ii) Histamine Production During Secondary MLC Performed with Stimulating Cells with Partial Homology at the H-2 Region with Immunizing Cells. Table VI shows that partial homology at the H-2 region between immunizing and stimulating cells is sufficient to induce an increase in histamine production during the secondary MLC. Homology at IC-S-G and D loci is sufficient (Table VI, combinations 2 and 3) to induce the increase in histamine production, whereas homology at IC-S and G loci is insufficient (Table VI, combination 4). Similarly, homology at IE-IC-S-G and D loci (Table VI, combination 6) is also sufficient, whereas identity at IE-IC-S and G loci (combination 11) is insufficient. This suggest the importance of the homology at D locus and this is confirmed in combination 5, where 70% of the response obtained with immunizing cells is obtained with a stimulating cell having only the H-2D region in common with donor cells. Homology at K-IA-IB-IJ and IE loci is also sufficient to obtain an increase in histamine production identical to that obtained with immunizing cells (Table VI, combination 8 and 10), but homology at IA-IB and IJ loci (Table VI, combination 12) induces only 18.5% of the maximal response. This suggests the importance of the homology at K locus.

Evidence for the Production of HCSF during Secondary MLC. 48-h dialyzed secondary MLC supernates (<2 ng/ml histamine) were tested for their HCSF activity. Incubation of normal spleen cells for 48 h with dialyzed supernate of mixed cultures between allograft donor and recipient cells induced a strong increase in histamine production

TABLE V

Histamine Production in Secondary MLC Performed with Third-Party Cells Differing at the Whole H-2 Region with Immunizing Cells

	Percentage of increase in histamine production* after 48 h of culture with spleen cells from				
	B10 ($H\text{-}2^b$) (8)	B10D2 ($H\text{-}2^d$) (8)	B10BR ($H\text{-}2^k$) (8)	B10S ($H\text{-}2^s$) (4)	B10M ($H\text{-}2^f$) (3)
Spleen cells from B10 ($H\text{-}2^b$) mice bearing skin graft from					
B10D2 ($H\text{-}2^d$) mouse	0	100	23.5 ± 6.5	5.3 ± 2.6	3.3 ± 2.8
B10BR ($H\text{-}2^k$) mouse	0	7.5 ± 2.4	100	12.8 ± 6.0	12.3 ± 4.2

Primary MLC was performed with B10 mice bearing syngeneic skin graft. Number of experiments is in parenthesis.

* The percentage of increase in histamine production was calculated with the following formula:

$$\text{percentage} = \frac{\text{percentage of increase in histamine production during secondary MLC performed with tested cells} - \text{percentage of increase in histamine production during primary MLC performed with tested cells}}{\text{percentage of increase in histamine production during secondary MLC performed with immunizing cell} - \text{percentage of increase in histamine production during primary MLC performed with immunizing cell}} \times 100.$$

TABLE VI

Histamine Production during Secondary MLC Performed with Stimulating Cells Bearing Partial Homology at the H-2 Region with Immunizing Cells

Combinations	Recipient	Donor	Stimulating cells	H-2 homology between donor and stimulating cells	Number of experiments	Percentage of increase* in histamine production after 48 h of incubation
1	B10	B10D2	B10D2	Total	8	100
2	B10	B10D2	B10A	IC-S-G-D	8	90.1 ± 13.6
3	B10	B10D2	B10A5R	IC-S-G-D	5	79.6 ± 6.1
4	B10	B10D2	B10A2R	IC-S-G	5	6 ± 1.9
5	B10	B10D2	B10HTT	D	5	70.4 ± 10.6
6	B10	B10D2	B10AQR	IE-IC-S-G-D	4	86.5 ± 8
7	B10	B10BR	B10BR	Total	8	100
8	B10	B10BR	B10A	K-IA-IB-IJ-IE	8	97.5 ± 7.7
9	B10	B10BR	B10A5R	IJ-IE	5	4.2 ± 2.2
10	B10	B10BR	B10A2R	K-IA-IB-IJ-IE	5	106.6 ± 14.5
11	B10	B10BR	B10HTT	IE-IC-S-G	5	6.8 ± 4
12	B10	B10BR	B10AQR	IA-IB-IJ	4	18.5 ± 9.1

* Percentage of increase in histamine production is calculated by estimating the response obtained with immunizing cells as 100% (see Table IV).

when compared with that obtained after a 48-h incubation with control supernates (mean values of increase percentage from 22 experiments: 170.3 ± 21.7%).

Such an effect of HCSF-containing supernate is not detected after 6 h of incubation, but appears after 24 h, reaches its maximum at 48 h, and remains at the same level after 72 h.

HCSF activity may be detected by using spleen, bone marrow, blood, or peritoneal cells as targets but not with lymph node and thymus cells (Table VII). As the spontaneous histamine release from peritoneal cells is higher than that of spleen or bone marrow cells (294.4 ± 81.1 ng/ml, 29.2 ± 5.9 ng/ml, and 117.7 ± 20.4 ng/ml, respectively), the percentage increase induced by HCSF on peritoneal cells is less than with spleen or bone marrow cells (42.6 ± 15.4% against 170.3 ± 21.7% and 305.5 ± 25.6%, respectively).

The activity is concentration dependent because it is usually lost by a 30- to 60-fold dilution of the supernate.

With bone marrow or spleen cells as target cells of HCSF activity, it can be seen in Table VIII that the effect of HCSF is not major histocompatibility complex (MHC) restricted, as HCSF obtained from C57BL/6 mouse (H-2^b) can increase histamine production from target cells bearing different MHC (H-2^d, H-2^k, or H-2^s). On the other hand, mouse HCSF cannot act on rat or guinea-pig spleen and bone marrow cells (Table VIII).

TABLE VII

HCSF-containing Supernate Effect on Histamine Production by Various Kinds of Cells

	Histamine production increase* after HCSF-containing supernate treatment on 10^7 cells from					
	Spleen	Bone marrow	Blood	Peritoneal cavity	Lymph nodes	Thymus
			ng/ml			
Mean values from six experiments ± SEM	89 ± 9	578 ± 76	166 ± 31	140 ± 61	6 ± 3	4 ± 2

* Difference between histamine production after HCSF-containing supernate and that obtained after control supernate treatment.

TABLE VIII

HCSF Activity is Not MHC-restricted but Cannot Induce Modification in Xenogenic Cells

H-2^b HCSF activity* tested on	Percentage of histamine increase by	
	Spleen cells	Bone marrow cells
H-2^b cells (C57BL/6 and B10) (9)‡	124.4 ± 38.2	383.3 ± 53.9
H-2^d cells (DBA/2 and B10D2) (9)	118.1 ± 38.7	252.8 ± 46.1
H-2^k cells (CBA and B10BR) (9)	137.6 ± 28.4	272.3 ± 69.7
H-2^s cells (SJL and B10S) (4)	106.5 ± 9.5	324 ± 47.4
Rat cells (4)	6.25 ± 6	4.5 ± 2.5
Guinea pig cells (2)	5 ± 1	0

* HCSF has been obtained during secondary MLC between DBA/2- (H-2^d) grafted C57BL/6 (H-2^b) and DBA/2 (H-2^d).

‡ Number of experiments is in parenthesis.

Effect of HCSF on Bone Marrow Cells

To study the HCSF mechanism of action, we have chosen bone marrow cells as target cells because they are the most sensitive to HCSF (Table VII).

EFFECT OF HCSF ON HISTAMINE RELEASE. Before culture, bone marrow cells contained a very small amount of histamine. Incubation with HCSF increases the total histamine content. As shown in Table IX, HCSF induces an increased histamine production not only in supernates but also within cells (means values of four experiments: 62.6 ± 2.5 ng in HCSF-treated cells instead of 17.4 ± 4.1 ng in control-treated cells).

EFFECT OF HCSF ON CONVERSION OF HISTIDINE INTO HISTAMINE. Cultures of bone marrow cells were performed in the presence of 4 μCi of L-[2-^{14}C]histidine (i.e., 72 nmol). After a 48-h incubation, labeled histamine was determined in the supernate of bone marrow cells treated with control or HCSF-containing supernate. The results of a typical experiment reported Fig. 2, clearly show that HCSF induces an increase in the transformation of labeled histidine. 2.2% of total histidine (i.e., 10.4 nmol) is transformed into histamine during HCSF treatment instead of 0.67% (i.e., 3.2 nmol) during control treatment.

EFFECT OF HCSF ON HISTIDINE DECARBOXYLASE ACTIVITY. The increase of transformation of histidine into histamine after HCSF treatment strongly suggests that this factor acts by increasing histidine decarboxylase (HDC) activity. This has been confirmed by determining HDC activity in the lysate of bone marrow cells after control or HCSF-containing supernate treatment. HDC activity was weak, but a significant increase is induced by HCSF-containing supernate (at pH 7.4, mean values of six experiments were: 284 ± 45% of increase; at pH 6.6, 333 ± 179%), when compared with control supernate.

Location of HCSF Target Cells After Discontinuous Ficoll Gradient Separation. As shown in Table X, bone marrow cells from layers 2 and 3 were the most sensitive to HCSF activity, whereas layers 0, 1, 4, 5, and 6 showed minimal stimulation.

TABLE IX

Histamine Content of Bone Marrow Cell Culture after HCSF Treatment

	Exp.	Histamine content in		
		Cells	Supernate	Total
			ng	
Before treatment	1	6.2	2.6	8.8
20×10^6 bone marrow cells (in 2 ml)	2	6.6	3.8	10.4
	3	5.9	4	9.9
	4	4.7	2.4	7.1
After 48 h of control treatment	1	29	370	399
20×10^6 bone marrow cells (in 2 ml)	2	10.4	344	354.4
	3	13.8	458	471.8
	4	16.5	392	408.5
After 48 h of HCSF treatment	1	60.6	1,487	1,547.6
20×10^6 bone marrow cells (in 2 ml)	2	57	1,180	1,237
	3	68.7	1,462	1,530.7
	4	64.3	618	682.3

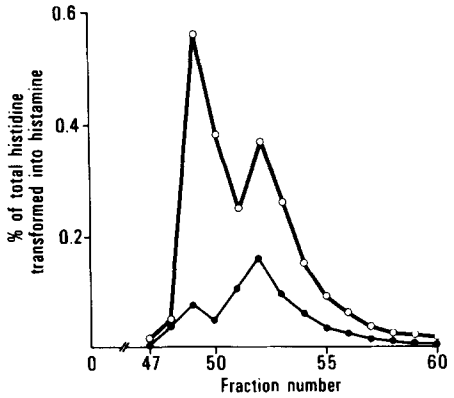

FIG. 2. Histamine formation from histidine by control or HCSF-treated cells. (○) Histamine formation by HCSF-treated cells; (●) histamine formation by control-treated cells.

TABLE X

Fractionation of Bone Marrow Cells by Ficoll Gradient Location of HCSF Target Cells

Layers	Percentage of overall recovered cells	Background of histamine production per 10^6 cells	Mean (± SEM) increase in histamine production per 10^6 cells incubated with HCSF-containing supernate
		ng	*ng*
0	0.26 ± 0.03	15 ± 5.4	1.2 ± 0.6
1	0.23 ± 0.03	34 ± 9	108 ± 20.4
2	0.63 ± 0.23	425 ± 71.2	1,194.6 ± 278.1
3	3.13 ± 1.08	389.6 ± 91.6	1,433.3 ± 346.5
4	13.57 ± 3.24	112.2 ± 38.8	378 ± 134.9
5	38.85 ± 3.76	33.4 ± 6	60.2 ± 15.3
6	43.43 ± 3.44	22 ± 3.8	27.2 ± 9.5

Results are the mean values ± SEM of seven experiments. After HCSF treatment the increase in histamine production by 10^6 nonfractionated bone marrow cells in these experiments is 147.5 ± 53.2 ng.

Morphological observations show that layers 2 and 3 contain some cells with an indented, bi-, or polylobulated nucleus, and have granules distributed throughout the cytoplasm. The granules are stained with alcian blue at pH 0.3 and toluidine blue (with metachromasy). In smears treated with ortho-phtaldialdehyde (intracellular detection of histamine), these granules show a yellow fluorescence.

These features may be considered as characteristic of basophilic cells. Such cells are sparce, but appear to be more numerous after HCSF-containing-supernate treatment (1–3%) when compared with control supernate treatment (<0.5%).

Physicochemical Characteristics of HCSF. The HCSF activity was entirely retained in supernates incubated at 56°C for 2 h and also at 80°C for 10 min (after centrifugation to eliminate the precipitate) (Fig. 3). Ultrafiltration on Amicon filter XM 300 and XM 100 did not abolish HCSF activity in the ultrafiltrate (mean values of three experiments: 239 ± 65% and 218.7 ± 20% of increase production of histamine by ultrafiltrate on XM 300 or XM 100, versus 307.2 ± 24.3% by nonfiltered supernate, respectively). Ultrafiltration on Amicon filter XM 50 retained HCSF activity in the

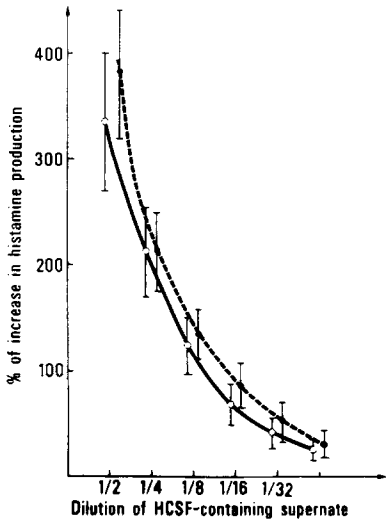

FIG. 3. Effect of heat treatment (80°C for 10 min) on HCSF activity. Mean values of five experiments ± SEM. (○) Percent of increase in histamine production by normal bone marrow cells in presence of HCSF-containing supernate without heat treatment; (●) same as above in presence of HCSF-containing supernate previously heated for 10 min at 80°C.

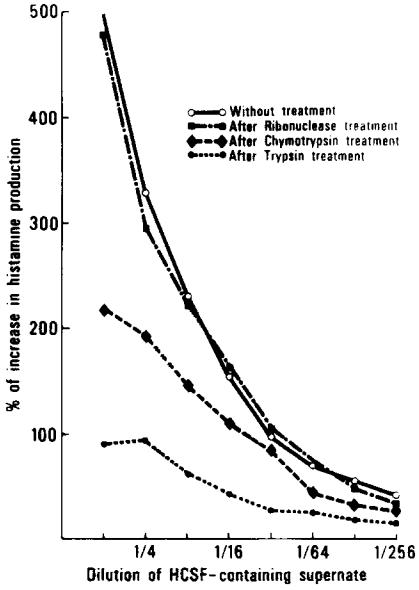

FIG. 4. Effect of enzyme treatment on HCSF activity. A typical experiment. The same kind of results have been obtained in three other experiments.

medium upper filter (174.3 ± 58% of histamine increase by medium upper filter against 24 ± 2% ultrafiltrate). As shown in Fig. 4, treatment with trypsin abolished HCSF activity. Chymotrypsin treatment strongly diminished HCSF activity, whereas ribonuclease treatment had no effect.

Discussion

Many substances are produced during MLC between allograft donor and recipient. They include a series of lymphokines, such as the migration inhibitory factor (22) and the macrophage-activating factor (23, 24), and more common substances such as prostaglandins (25) and urea and ornithine (26). In this paper, we demonstrate that histamine may also be found in large amounts. Histamine production is greatly increased during culture of allograft recipient spleen cells in the presence of immunizing cells (secondary MLC) as compared with that found in primary MLC (i.e., without previous allograft sensitization). The increase in histamine production observed during secondary MLC may be explained by the action of a soluble factor produced during culture, because the dialyzed secondary MLC supernate is able to induce increased histamine production from normal spleen or bone marrow cells. We suggest that this mediator be termed: histamine-producing cell stimulating factor (HCSF).

The HCSF-producing cells are probably T lymphocytes because: (*a*) treatment of recipient spleen cells by monoclonal anti-mouse Thy-1.2 antibody strongly diminishes histamine production during specific MLC and (*b*) concanavalin A stimulation of normal spleen cells induces HCSF production (M. Dy, B. Lebel, P. Kamoun, and J. Hamburger, manuscript in preparation). The increase in histamine production during secondary MLC requires specific stimulation with cells bearing, at least partly, the H-2 antigens of immunizing cells. Partial homology at the H-2 complex between immunizing and stimulating cells is sufficient to induce the phenomenon. However, studies using recombinant congenic mice demonstrate that such an homology has to be coded by K and D loci. In that respect, HCSF production differs from proliferative responses during secondary MLC, because an increase in [^{3}H]thymidine incorporation can be fully triggered by determinants coded by the I region of the H-2 complex (27, 28). Conversely, there is an immunogenetic similarity between HCSF production and lymphocyte cytotoxicity: both require K- and D-coded determinant recognition (29). Whatever the exact subclasses of T lymphocytes producing HCSF, our results strongly suggest that this factor is a lymphokine. Like other factors produced by lymphocytes, HCSF is still produced by mitomycin-C-blocked lymphocytes and its effect is not MHC restricted.

Studies of the mechanism of action of HCSF on bone marrow cells demonstrate that it induces an increase in histamine synthesis. This conclusion is based on the following facts: (*a*) the amount of intracellular histamine of bone marrow cells before culture is extremely low; (*b*) the total histamine content (cells and supernates) is much greater in HCSF-treated cells than in cells treated by control supernates, (*c*) HCSF induces an increase in the amount of labeled histidine transformed into histamine; and (*d*) HDC activity is increased in the homogenate of HCSF-treated bone marrow cells when compared with that of control cells.

Although it is clear that HCSF induces an increase in histamine synthesis, at least two possibilities remain open: (*a*) HCSF may act on its target cell by increasing its HDC activity or (*b*) HCSF may increase the number of cells that contain HDC. That mitomycin C blocks neither the increase in histamine production during secondary MLC nor the effect of HCSF on bone marrow cells (M. Dy, B. Lebel, P. Kamoun, and J. Hamburger, manuscript in preparation) would suggest that the increase in histamine production does not result from a proliferative response. However, other

data suggest that HCSF might act as a factor of maturation or even proliferation of precursor cells with initially low histidine decarboxylase activity. These data are: (*a*) the target cell is clearly much more abundant in bone marrow than in other sites; more generally, the intensity of the observed effect is well correlated with the distribution of mast cell precursors in hematopoietic and lymphopoietic tissues of mice (30, 31) and (*b*) fractionation of bone marrow cells on a discontinuous Ficoll gradient shows that cells from layers 2 and 3 (the less-dense cells) are the most sensitive to HCSF. In these layers, morphological studies show the presence of cells having the features of immature cells with granules; these cells could be precursors of basophils or mastocytes. They are more numerous after HCSF supernate treatment for 48 h, when compared with control treatment.[2]

Some factors produced by leukocytes and which trigger the release of histamine from basophils have been described (32–34). These factors differ from HCSF in several significant ways, including their mechanism of action (an increase in histamine release instead of an increase in histamine synthesis), their time-course of histamine production, and their apparent molecular weights.

Recently, a factor (basophilopoietine) produced by antigenic or mitogenic lymphocyte stimulation in guinea-pigs and increasing basophil production in bone-marrow has been described by Denburg et al. (35). The possible relation between HCSF, basophilopoietine (35) and others lymphomononuclear cell-derived factors which stimulate the in vitro differentiation of bone marrow precursor cells (36–38) will have to be further investigated.

In the allograft situation, the production of HCSF may explain the number of basophils found among cells infiltrating allografts in the guinea-pig (39) and the increase of histidine decarboxylase found in skin allografts at the time of rejection (4) because the maximum level of HCSF production coincides with skin rejection (Fig. 1). The local production of histamine induced by HCSF could play a double role in allograft rejection. First, it may contribute to the alteration in capillary permeability, which is known to be important in the mechanism of rejection. Second, it might interfere with the regulation of the immune process. In effect, it is well established now that histamine interferes with the lymphocyte response. The following hypothesis may be presented: Sensitized lymphocytes infiltrating the graft secrete lymphokines, including HCSF. This factor would act on basophil or mastocyte precursors. Histamine thus released locally stimulates suppressor T lymphocytes that would, in turn, inhibit the lymphokine production and the other steps of the lymphocyte response (40). The phenomenon may then represent a local feedback immunoregulation, limiting the response both in duration and extent. Such an hypothesis concerning the allograft model can be compared with a similar hypothesis suggested in the case of delayed-type hypersensitivity reactions (40).

Summary

Histamine production is greatly increased during culture of allograft recipient spleen cells in the presence of immunizing cells (secondary mixed leukocyte cultures [MLC]) as compared to that found in primary MLC (i.e., without previous allograft).

[2] Since this paper was written, we have also found that long-term-culture experiments of the above-mentioned layers 2 and 3 in the presence of HCSF-containing supernates induce a large proliferation of mastocytes.

This phenomenon appears after 24 h of culture and reaches its maximum at 48 h. Optimal increased histamine production is observed when MLC is performed with spleen cells removed from mice during rejection. This increased production of histamine during secondary MLC results from the action of a lymphokine: the histamine-producing cell stimulating factor (HCSF). This factor is released by T lymphocytes. Its production requires specific stimulation of the recipient lymphocytes because increase in histamine production during secondary MLC can be only observed when recipient cells are cultured with stimulating cells bearing at least one homology at K or D loci with immunizing cells. HCSF acts on a cell which is present in bone marrow, spleen, blood, and peritoneal cells but absent in thymus or lymph node cells. This target cell is found in the less-dense layer of a discontinuous Ficoll-gradient of bone marrow cells. HCSF is heat stable, destroyed by trypsin treatment, and has a molecular weight between 50,000 and 100,000. It acts on its target cells by increasing histidine decarboxylase activity.

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