

Oestradiol enhances *in vitro* the histamine release induced by embryonic histamine-releasing factor (EHRF) from uterine mast cells

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The relationship between maternal hormones and factors secreted by the implanting embryo is still controversial. We have analysed the *in-vitro* effect of oestradiol and human embryo-derived histamine-releasing factor (EHRF) on histamine release from rat uterine mast cells. Rat uterine mast cells which were preincubated with oestradiol and then challenged with human EHRF gave histamine release values two- to threefold higher than those without preincubation. The enhancement observed was time- and temperature-dependent. A similar enhancement was obtained with human sensitized basophils but not with rat peritoneal mast cells. Oestradiol, used as a direct challenge, did not induce any histamine release from either rat uterine or peritoneal mast cells, or from human sensitized basophils. Oestradiol preincubation also enhanced the histamine release induced by anti-IgE but did not enhance the histamine release induced by substance P or compound 48/80, two secretagogues that are not mediated by IgE. Moreover, uterine fragments derived from rats at various oestrus phases, with different amounts of endogenous oestrogen, were challenged *in vitro* with EHRF. The release of histamine by mast cells was higher at the proestrus and preimplantation phases than at dioestrus. All these findings suggest that the interaction of oestradiol with rat uterine mast cells was capable of enhancing *in vitro* the histamine releasing effect of EHRF.

Key words: EHRF/IgE/oestradiol/uterine mast cell

Introduction

Pregnancy depends on synchronized events, orchestrated by an early maternal–embryonal dialogue (Adinolfi, 1989). The local uterine changes occurring at the site of blastocyst implantation suggest that embryonic signals could be very important (Hore and Mehrotra, 1988; Weitlauf, 1989). The maternal–embryonal dialogue is very likely to be controlled by a sequence of

neuro-hormonal events interacting with the immune system (Hall *et al.*, 1989).

Previous results obtained by our group showed that embryonic histamine-releasing factor (EHRF) secreted by the human preimplantation embryo (Cocchiara *et al.*, 1987a,b, 1988a) interacts with rat uterine mast cells *in vitro* causing release of histamine (Cocchiara *et al.*, 1988b). Several studies (Brandon and Bibby, 1979; Hore and Mehrotra, 1988; Cocchiara *et al.*, 1988b) report an increase of the number of rat uterine mast cells and of the total content of uterine histamine, reaching a maximum during the preimplantation stage and declining when embryo implantation is established. Moreover, the oestradiol concentration increases during the preimplantation stage in rats (Yoshinaga *et al.*, 1969; Findlay, 1983; Hodgen and Itskovitz, 1989), and some studies suggest that the oestradiol is responsible for the degranulation of rat uterine mast cells and for the release of histamine *in vivo* (Spaziani and Szego, 1959; Green *et al.*, 1964; Ferrando and Nalbandon, 1968; Gibbons and Chang, 1972). Conflicting results, as reviewed by Weitlauf (1989), are reported on the *in-vivo* role of histamine and mast cells, since Shelesnyak (1952) suggests that this vasoactive amine can play a key role in implantation and Spaziani and Szego (1959) suggest a decrease in the mast cell population and histamine content as a result of the rise in circulating maternal oestrogen. A role of uterine mast cells during implantation could be supported by recent data on the molecules secreted by mast cells in addition to histamine, such as cytokines (Galli *et al.*, 1991; Fay and Grudzinskas, 1991) and prostaglandins (Kennedy, 1977; Toder *et al.*, 1984; Loke, 1989).

Observations of this sort have led us to determine whether oestradiol can influence degranulation of rat uterine mast cells and whether a factor secreted by the implanting embryo could be involved in this process. The availability of EHRF preparations and of uterine mast cells makes it possible to test this hypothesis *in vitro*.

Materials and methods

Buffers

HEPES-buffered Tyrode's solution (10 mM HEPES, 137 mM NaCl, 5.6 mM glucose, 2.7 mM KCl, 0.4 mM NaH₂PO₄, 1 mM CaCl₂, 1 mM MgCl₂) with 0.3% bovine serum albumin (BSA) titrated to pH 7.4, was used for the histamine release experiments and for the collection of peritoneal cells. The same solution but free of calcium and magnesium ions, was used for tissue washing. Tris–ACM buffer (0.025 M Tris, 0.12 M NaCl,

6×10^4 M CaCl_2 , 0.001 M MgCl_2 , pH 7.4) with added 0.3% BSA was used for the histamine release assay from atopic blood basophils. Phosphate-buffered saline (PBS), 0.05 M pH 7.9, was used in the histamine single isotope microassay.

Materials

Oestradiol was prepared at a concentration of 200 $\mu\text{g/ml}$ (Sigma Chemical Co. Ltd, St Louis, MO, USA) in a stock solution containing Tyrode's buffer and 60 mg/ml BSA. Sheep anti-rat IgE was purchased from Tago Inc. (Burlingame, CA, USA). Both of the anti-IgE were used diluted by 1:100. S-Adenosyl-L-methyl-[^3H]methionine was purchased from Amersham Radiochemical Centre, UK. EHRF was isolated from human embryo culture supernatants by means of a heparin–Sephacryl column and was prepared in our laboratory as previously described (Cocchiara *et al.*, 1987a, 1988a) and used at a concentration of 1 mg/ml. Briefly, 10 ml obtained from 10 human embryo culture media (grown *in vitro* during an in-vitro fertilization programme) were loaded onto a heparin–Sephacryl column. After washing, the EHRF activity was eluted with 0.5 M NaCl. The activity was tested by histamine release assay, using human basophils from atopic donors. Compound 48/80 was obtained from Sigma and stored as a stock solution of 100 mg/ml in 1% dimethyl sulphoxide (DMSO).

Animals and tissue source

Wistar female rats (8 weeks old) were purchased from Morini (Reggio Emilia, Italy). The oestrus cycle was determined daily by analysis of vaginal smears for at least 9 days. The dark–light period during which mating occurred was designated night 0 of pregnancy, followed by day 1 (when mating plugs or vaginal spermatozoa were observed). The rats were killed and uteri were removed during different phases of the oestrus cycle or on different days of pregnancy, placed in cold Tyrode's solution, cleaned from adherent connective tissue and cut into pieces of 50 mg wet weight by scissors. Uterine fragments were washed in Ca^{2+} - and Mg^{2+} -free Tyrode's solution.

The peritoneal mast cells were collected by washing the peritoneal cavity with Tyrode's buffer supplemented with 0.3% BSA. They were washed in the same buffer and diluted to a final concentration of 350 000/ml. The basophils were obtained from venous blood of atopic donors, with their permission.

Histamine release experiments

The effect of histamine secretagogues on rat uterine fragments of peritoneal mast cells and human atopic blood basophils was determined. Rat uterine fragments were washed in Tyrode's buffer, 0.3% BSA final concentration. The samples were pre-warmed for 10 min at 37°C and incubated at 37°C in Eppendorf tubes containing 20 μl of each secretagogue, EHRF, anti-IgE or buffer alone. After 30 min, the reaction was stopped by addition of 300 μl cold Tyrode's buffer. Rat peritoneal mast cells were treated as reported previously (Cocchiara *et al.*, 1988b). A 200 μl volume of peritoneal cells at 350 000/ml was incubated in Tyrode's solution with various secretagogues (anti-IgE, EHRF, substance P, compound 48/80).

Aliquots of 200 μl of whole human atopic heparinized blood were incubated with different secretagogues in 20 μl of Tris–

ACM, 1 \times buffer. All the mixtures incubated at 37°C for 30 min were centrifuged in a Heraeus Sepatech Biofuge 17RS and triplicate aliquots of 20 μl of plasma were then removed for the histamine assay.

When experiments with oestradiol were performed, uterine or peritoneal mast cells or basophils were preincubated with oestradiol (1 $\mu\text{g/ml}$) for 30 min and then challenged with the secretagogue for 30 min. As a control, cells were preincubated with oestradiol for 30 min and challenged with buffer alone for 30 min.

Histamine release assay

Histamine was assayed by a single isotope enzymatic microassay, as previously described (Taylor *et al.*, 1980), by using 500 mCi/mmol S-adenosyl-L-methyl-[^3H]methionine (SAM) and a crude preparation of rat kidney histamine-methyl-transferase (HMT) (Shaff and Beaven, 1979), as previously described (Taylor *et al.*, 1980; Cocchiara *et al.*, 1988a).

To each triplicate aliquot of supernatant (20 μl) obtained after release experiments, 10 μl of HMT–SAM solution was added and the tubes, after a gentle mixing, were incubated at 37°C for 1 h. The reaction was stopped with 10 μl of 1 N NaOH, and the [^3H]methyl-histamine formed was extracted into 250 μl of chloroform. After centrifugation at 3000 g for 5 min, the aqueous phase was removed and the organic phase washed with 100 μl of NaOH (1 M) saturated with NaCl. After centrifugation, 150 μl of the organic phase was transferred to a scintillation vial and evaporated to dryness. The liquid scintillator was added and the bound radioactivity determined in a Beckman counter. The total histamine was determined by boiling the cells or the tissue fragments for 10 min with 0.05 M phosphate buffer (pH 7.9). Spontaneous release was calculated by incubating the cells or the tissue with buffer alone. The level of histamine release was expressed as a percentage of the total histamine after subtraction of the level of spontaneously released histamine. The enhancement of histamine release was calculated using the following formula: (histamine release with stimulus plus oestradiol – histamine release with stimulus)/total histamine content.

Statistical analysis

Data were expressed as mean \pm SEM of at least four repeated experiments. The experiments were performed in triplicate and the intra-assay SEM did not exceed 5%. One-way analysis of variance (ANOVA) was used to determine differences between groups. When differences existed, the ANOVA analysis was followed by the two-tailed (unpaired) Student's *t*-test.

Results

Oestradiol-induced enhancement of histamine release in response to stimulation with anti-IgE

The effect of oestradiol on histamine release was initially analysed using human basophils and anti-human IgE since this is a convenient experimental model. Kinetic experiments showed that oestradiol preincubation enhanced the histamine release in response to stimulation with anti-IgE in a time-dependent manner. The enhancement obtained after 60 min of oestradiol pre-

incubation was twofold higher (50%) than after preincubation for 0 min (27%) (Figure 1). The same cells, preincubated for different time periods with buffer instead of oestradiol released the same amount of histamine on challenge with anti-IgE (Figure 1). Histamine release was not observed when the basophils were preincubated with oestradiol but without any anti-IgE challenge (Figure 1). The statistical significance of the oestradiol enhancement was evaluated in experiments performed with basophils from 12 atopic subjects. The variability between the group means shown in Table I (anti-IgE and oestradiol + anti-IgE) was significantly larger than the average variability within the different treatment groups. There were very significant differences among the groups ($P = 0.0023$). Moreover, analysis using ANOVA was identical to results of the unpaired two-tailed t -test.

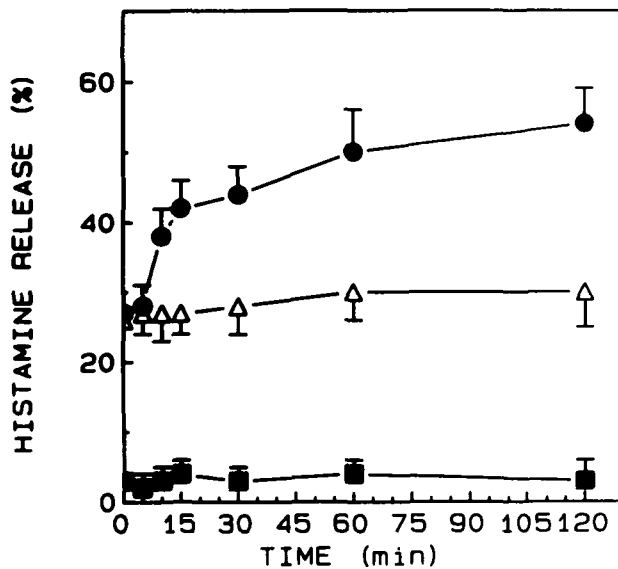


Fig. 1. Time-dependent enhancement of histamine release by oestradiol from leukocytes (human basophils). The cells were preincubated with 1 μ g/ml of oestradiol (\bullet) or buffer (Δ) for various times and then stimulated with anti-IgE (1:100) for 30 min. The values of histamine release obtained by challenge with oestradiol are also reported (\blacksquare). Data are the mean \pm SEM of four separate experiments.

Similar results were obtained using rat peritoneal mast cells (data not shown) and rat uterine fragments as the source of mast cells instead of human basophils.

Three representative experiments showing significant enhancement of histamine release upon preincubation with oestradiol and subsequent challenge with anti-IgE are reported in Table II ($P < 0.01$).

Oestradiol-induced enhancement of histamine release in response to stimulation with EHRF

The rat mast cells and the human basophils were preincubated with oestradiol and then challenged with EHRF. The uterine mast cells obtained from 30 rats gave a histamine release induced by EHRF of $14 \pm 4\%$. This release was always enhanced by preincubation with oestradiol, giving a value of $26 \pm 7\%$ (Figure 2). Human basophils from seven atopic donors showed histamine release values when challenged with EHRF of $15 \pm 5\%$, which increased to $37 \pm 9\%$ when preincubated with oestradiol. The peritoneal mast cells did not release histamine when challenged with either EHRF or oestradiol + EHRF. The standard deviation among the values obtained from different subjects was quite high, suggesting that each subject had a different sensitivity. However, the values obtained in the presence of oestradiol were significantly higher than the controls without the oestradiol preincubation ($P < 0.01$).

To evaluate whether there was any correlation between the in-vitro histamine release results and the in-vivo oestrogen variation, the following experiments were performed. Two different phases of the reproductive cycle were analysed: proestrus, when the oestradiol concentration was maximal and dioestrus, when oestradiol is absent or at a very low level. In addition, we analysed day 4 of pregnancy (preimplantation stage), when the oestradiol reaches the maximum concentration during pregnancy. At proestrus, the histamine release induced by anti-IgE and EHRF was $16 \pm 3\%$ and $18 \pm 4\%$ respectively. These values were higher than the values obtained in dioestrus when the histamine release by either anti-IgE or EHRF challenge was only 6%. At the preimplantation stage, the histamine release induced by anti-IgE was $16 \pm 4\%$ and by EHRF $31 \pm 6\%$

Table I. Enhancement of histamine release by basophils challenged with anti-IgE, with or without preincubation with oestradiol

Donors	Spontaneous histamine release ng/ml \pm SEM	Total histamine ng/ml \pm SEM	Anti-IgE ng/ml \pm SEM (%)	Oestradiol + anti-IgE ng/ml \pm SEM (%)	Enhancement (%)
1	16 \pm 1.3	92.7 \pm 5	29.55 \pm 3 (17)	45.5 \pm 3.2 (38)	17
2	33 \pm 2	147 \pm 3.2	79.6 \pm 4.2 (40)	124 \pm 9.6 (80)	30
3	24.2 \pm 4	249 \pm 10.5	44.8 \pm 2.5 (9)	103.3 \pm 11.1 (35)	23
4	22 \pm 5.2	170 \pm 9.7	121 \pm 7.3 (66)	138 \pm 9.5 (78)	10
5	20 \pm 4.3	163 \pm 15.3	73.4 \pm 4.1 (37)	116.8 \pm 8.7 (68)	26
6	11.7 \pm 1.2	136 \pm 9.2	32 \pm 1.8 (16)	88 \pm 5.2 (61)	41
7	26 \pm 1.5	269 \pm 11.2	80.3 \pm 6.2 (22)	113 \pm 9.3 (35)	12
8	28 \pm 1.3	143 \pm 6.1	91.3 \pm 5.5 (55)	126 \pm 8 (85)	24
9	24 \pm 1.6	148 \pm 5.8	62.3 \pm 4.9 (30)	92.5 \pm 4 (55)	20
10	12 \pm 1.1	114 \pm 6.9	50.2 \pm 3.2 (37)	70.4 \pm 8.2 (57)	18
11	7.4 \pm 0.9	115 \pm 7.4	43.8 \pm 2.8 (45)	72.5 \pm 4.3 (59)	25
12	11 \pm 1	129 \pm 5.8	40.3 \pm 3.4 (27)	60.7 \pm 5.2 (41)	16

Values of histamine release are expressed as ng/ml (mean \pm SEM of triplicate experiments). The percentage histamine release [indicated as (%)] with oestradiol plus anti-IgE was significantly higher ($P < 0.01$) than data obtained with anti-IgE alone.

Table II. Effect of oestradiol preincubation on the enhancement of histamine release induced by anti-IgE from uterine fragments

Experiment	Oestradiol preincubation	Anti-IgE release	Histamine release, %	± SEM
I	+	-	1.5	± 0.07
	-	+	18.6	± 1.5
II	+	+	34.1	± 2.7
	+	-	1.7	± 0.9
	-	+	15.1	± 1.2
III	+	+	30.2	± 2.2
	+	-	1.3	± 0.07
	-	+	8.6	± 0.8
	+	+	22.1	± 1.8

Uterine fragments were (+) or were not (-) preincubated with 1 µg/ml oestradiol for 30 min and then challenged with anti-IgE. Results are the mean ± SEM of triplicate experiments. The histamine release obtained in the presence of oestradiol was significantly higher ($P < 0.01$) than the control without oestradiol.

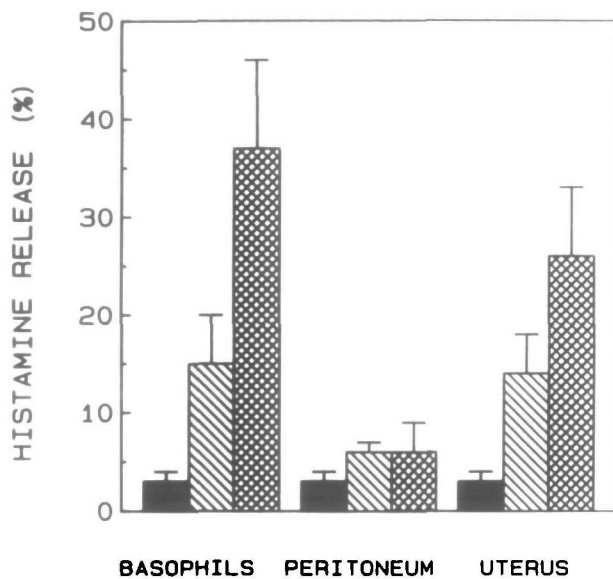


Fig. 2. Effect of oestradiol preincubation on EHRF-induced histamine release from basophils, peritoneal mast cells and uterine fragments. Cells were preincubated (▩) or not preincubated (▨) with 1 µg/ml oestradiol for 30 min and stimulated with EHRF for 30 min. Values of histamine release obtained with oestradiol alone (■) are also shown. The data are expressed as mean ± SEM of different experiments. The values from basophils and uterine fragments of EHRF-induced histamine release after preincubation with oestradiol were significantly higher than the control without preincubation ($P < 0.01$).

(Figure 3). As a control, oestradiol used alone did not induce any histamine release from the uterine fragments (Figure 3).

In experiments designed to understand the mechanism(s) involved in the enhancement of the histamine release by oestradiol, we found that the effect was temperature dependent in the same fashion as the anti-IgE-induced histamine release (Table III).

Histamine release induced by substance P and compound 48/80 was not enhanced by oestradiol

Two non-IgE mediated secretagogues, such as substance P and compound 48/80, were used instead of the anti-IgE, to evaluate

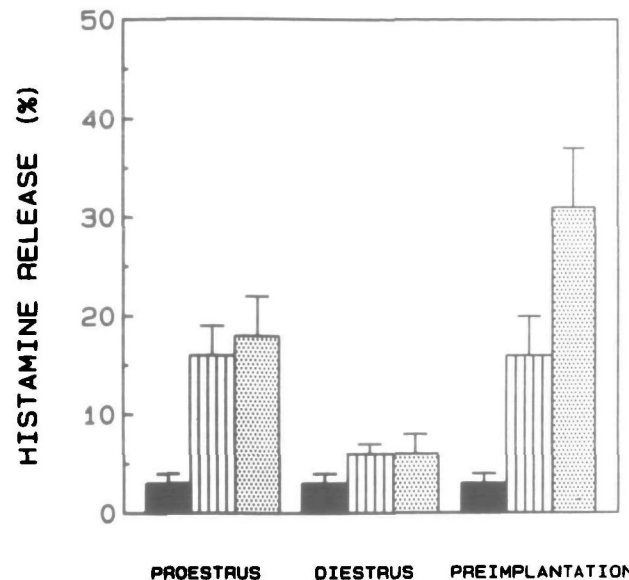


Fig. 3. Effect of oestradiol on histamine release by uterine fragments during the different phases of the reproductive cycle. Uterine fragments at proestrus, diestrus and preimplantation phase (day 4 of pregnancy) were stimulated with oestradiol (■), anti-IgE (▨) or EHRF (▩) for 30 min. The results are the mean ± SEM of five different experiments.

Table III. Different effects of oestradiol preincubation on peritoneal mast cells stimulated with anti-IgE, substance P or compound 48/80

	Histamine release	
	(ng/ml ± SEM)	(%)
Spontaneous release (Tyrode's)	6.25 ± 0.5	-
Oestradiol (1 µg/ml)	7.3 ± 0.9	1.2
Tyrode's + anti-IgE	27.0 ± 1.8	25.0
Oestradiol (1 µg/ml) + anti-IgE	46.0 ± 2.3	48.0
Oestradiol (1 µg/ml) (4°C) + anti-IgE (4°C)	6.0 ± 0.4	0
Oestradiol (1 µg/ml) (4°C) + anti-IgE (37°C)	25.6 ± 0.5	24
Substance P (25 µM)	54.6 ± 2.2	59.0
Oestradiol (1 µg/ml) + substance P (25 µM)	41.6 ± 1.9	43.0
Compound 48/80 (10 µg/ml)	39.0 ± 0.7	40.0
Oestradiol (1 µg/ml) + compound 48/80	37.5 ± 1.4	38.0
Total histamine	88.0 ± 2.1	

Peritoneal mast cells were or were not preincubated (37°C) with oestradiol (1 µg/ml) for 30 min and then challenged with anti-IgE, substance P or compound 48/80. Controls comprised cells preincubated with oestradiol at 4°C + anti-IgE at 4°C or with oestradiol at 4°C + anti-IgE at 37°C. The results are expressed as ng of histamine released per ml of assay ($n = 3$). The percentage of total histamine was calculated after subtraction of spontaneous release.

whether the IgE molecules were involved in the oestradiol-mediated enhancement. When different concentrations of oestradiol and substance P or compound 48/80 were used, no enhancement of histamine release was detected. These results are summarized in Table III where the results with only one concentration of oestradiol (1 µg/ml) and secretagogue are shown. The standard deviation among the results obtained from different individuals was quite high, suggesting that each had a different sensitivity. However, the values obtained in the presence of oestradiol were significantly higher than the controls made without oestradiol preincubation ($P < 0.001$).

The effect of oestradiol on the enhancement of mast cell histamine release induced by EHRF and anti-IgE was not observed when two non-IgE mediated secretagogues, such as compound 48/80 and substance P, were used. Therefore, these data suggest that the oestradiol was probably involved in the exposition of receptors of the IgE molecule present on the basophil and mast cell surface and specifically interacting with EHRF and anti-IgE. In addition, this finding provides evidence that a signal from the human embryo (EHRF) could interact with IgE molecules present on the surface of the maternal uterine cells.

Oestradiol, although capable of enhancing histamine release induced by specific secretagogues, was not capable of directly inducing histamine release *in vitro*. This result is in contrast with the *in-vivo* data reported in the literature. As is well known, the endogenous oestradiol concentration changes during the oestrus cycle. Several workers suggest that the mast cell population and the histamine content decrease as a result of circulating oestrogen (Levier and Spaziani, 1966; Gibbons and Chang, 1972).

A relationship between the number of uterine mast cells and the different phases of the reproductive cycle has been established *in vivo* (Gibbons and Chang, 1972; Brandon and Bibby, 1979; Brandon and Evans, 1983). The number increases from day 3 to day 4 of pregnancy and then decreases around the time of blastocyst attachment (De Feo, 1967; Brandon and Bibby, 1979).

Discussion

The present study demonstrates that oestradiol preincubation *in vitro* enhances the histamine-releasing action of EHRF as well as anti-IgE on human basophils and rat uterine mast cells. The histamine release from the uterine mast cells induced by EHRF was higher at the proestrus and preimplantation stages of the reproductive cycle than at dioestrus.

Previous studies made by our group showed that pre-embryos growing *in vitro* produce a histamine-releasing factor (EHRF) from the 2-cell to the 8-cell stage in humans (Cocchiara *et al.*, 1987a,b, 1988a,b), and from the 2-cell to the blastocyst in rats (preliminary results), and it was active as a histamine releaser in human basophils and rat uterine mast cells. The human and rat EHRF showed similar activity and there was cross-species reactivity. The latter is not surprising or unusual, since it has been reported in the literature and confirmed by several laboratories that IgE receptors in human cultured basophils bind both human and rodent IgE with comparable affinity (Ishizaka *et al.*, 1985).

The human EHRF, while capable of inducing histamine release from human basophils and rat uterine mast cells, was inactive when rat peritoneal mast cells were used as the target cells. A tentative explanation could be that the local environment or tissue-specific differences in differentiation might modify the cell surface specificity, resulting in mast cell heterogeneity, as reported in the literature (Befus, 1986).

The rat uterine mast cells preincubated with oestradiol and then challenged with human EHRF gave histamine release values which were two- to threefold higher than values obtained without preincubation. Brandon and Bibby (1979) observed that the number of mast cells increases during the pre-attachment phase of pregnancy to reach a maximum around the time of blastocyst

attachment and then decreases during the attachment phase of pregnancy. They claim that the reduction in mast cell number occurred both within and between attachment sites, suggesting the presence of a stimulus of maternal origin. However, more recently Hore and Mehrotra (1988) clearly demonstrated that the fall in the mast cell number in implanting sites was higher than in the interimplantation area. Therefore, a factor, or the presence of the blastocyst itself could cause the localized cell depletion.

The results reported in this paper strongly suggest that, *in vitro*, oestradiol used alone or as a secretagogue is completely inactive. Moreover, when uterine tissue fragments from rat uteri at different oestrus phases were used, different results were obtained. The endogenous oestradiol concentration was higher at proestrus and during the preimplantation stage and lower at dioestrus. When the uterine fragments containing mast cells were collected at proestrus or at the preimplantation stage and then challenged with EHRF *in vitro*, the resulting histamine release values were higher than at dioestrus stage. A tentative explanation is that the uterine mast cells in a milieu with a higher oestradiol concentration become more reactive on exposure to the EHRF challenge. The coincidence of the timing of the higher concentration of oestradiol during the preimplantation phase, the number of mast cells and the EHRF secretion by the preimplantation embryo, would suggest a probable *in-vivo* role of the combined action of oestradiol and EHRF. The high number of target uterine mast cells during the preimplantation stage would amplify the EHRF message sent by the implanting embryo, by the secretion of molecules contained in the mast cells. However, the percentage of enhancement of the histamine release by oestradiol, found at the preimplantation and proestrus phases, cannot depend on the higher mast cell concentration, since the number of mast cells found at proestrus was quite similar to or lower than at dioestrus.

Of particular interest could be the data reported in this paper on the *in-vitro* effect of oestradiol and EHRF on uterine mast cells, which may support an *in-vivo* role during embryo implantation of EHRF and uterine mast cells. The data could also help to clarify the relationship between maternal hormones and the factor(s) secreted by the implanting embryo.

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