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EFFECTS OF LYMPHOKINES AND MITOGENS ON A HISTAMINE DERIVATIVE-INDUCED INTRACELLULAR CALCIUM MOBILIZATION AND INOSITOL PHOSPHATE PRODUCTION

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Abstract—Histamine trifluoromethyl-toluidide derivative (HTMT), a novel immunosuppressive agent, stimulates H_1, H_2 and HTMT receptors in lymphocytes. HTMT receptors are different from the classical H_1, H_2 or H_3 receptors. Stimulation of HTMT receptors results in increased intracellular concentrations of calcium ($[Ca^{2+}]_i$) and inositol phosphate (IP) in human peripheral blood lymphocytes. In the present study, we investigated the effects of lymphokines [interleukin-4 (IL-4), interleukin-2 (IL-2)] and other pharmacologic agents [lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA)] on HTMT-induced Ca^{2+} and IP responses in non-rosetted cells. HTMT caused enhanced $[Ca^{2+}]_i$ and IP responses when the cells were pretreated with IL-4. The effects of IL-4 were concentration dependent and became maximal after the cells were incubated with IL-4 for 48 hr. Inhibitors of protein synthesis, but not of RNA synthesis, blocked the effects of IL-4 on HTMT-induced responses. LPS was more potent than IL-4 in augmenting Ca^{2+} mobilization induced by HTMT. However, the effects of LPS were not altered by inhibitors of either protein synthesis or RNA transcription. This indicated that LPS may act differently than IL-4 on the HTMT response. IL-2 and PMA did not affect HTMT-induced $[Ca^{2+}]_i$ and IP responses. The effects of IL-4 and LPS were agonist specific. They did not affect the Ca^{2+} mobilization induced by PAF. The data indicate that the response to HTMT can be regulated by IL-4 and LPS. Although the *in vivo* importance of these receptors is not yet clear, the receptor is likely a contributor to immune and/or inflammatory regulation.

Key words: histamine; lymphokines; $[Ca^{2+}]_i$; inositol phosphate; immunosuppressive agent; HTMT receptors

Histamine is a mediator and modulator of inflammation and immune responses [1–5]. Histamine regulates leukocyte function via both well defined and newly described cell surface receptors. We have reported previously the synthesis and pharmacologic effects of a series of derivatives of histamine that exhibit a broad spectrum of histamine-like pharmacologic activity [6, 7]. Some derivatives not only modulate the function of selected leukocytes but also have no pharmacologic action on non-lymphoid tissues. That is, they are tissue selective. The details of the immunologic characteristics of these congener derivatives of histamine have been described elsewhere [6, 7]. The most interesting derivative in this series, HTMT†, activates both H_1 and H_2 receptors in murine lymphocytes [6, 7]. While the activation of H_2 receptors results in an increase in cAMP accumulation, the activation of H_1 receptors in natural suppressor cells enhances their suppressive activity. The transducer associated with H_1 receptors in natural suppressor cells has not been identified. Furthermore, at higher concentrations, HTMT induces Ca^{2+} flux and IP turnover in human lymphocytes; these responses are not mediated by the classical H_1, H_2 or H_3 receptors [8]. Hence, we have called these receptors HTMT receptors.

The activation of HTMT receptors results in increases in intracellular calcium mobilization and formation of inositol phosphate in human peripheral blood lymphocytes. HTMT induces two phases of increase in the intracellular calcium concentration; (i) a rapid intracellular concentration peak within 10–60 sec of addition of drug, and (ii) partial recovery at 1–3 min after exposure to drug and a sustained moderate elevation that persists for more than 5 min. The EC_{50} is 1.9×10^{-5} M. Pretreatment of lymphocytes with HTMT results in desensitization to the compound, which recovers within 15 min after the cells are drug free. HTMT causes metabolism of phosphatidylinositol, resulting in phosphate inositol production. These data suggest that inositol triphosphate may be the second messenger for the mobilization of intracellular Ca^{2+} and that a specific binding site different from H_1, H_2 or H_3 receptors on leukocytes is the target for the compound [8].

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† Abbreviations: HTMT, histamine trifluoromethyl-toluidide derivative; $[Ca^{2+}]_i$, intracellular calcium level; IP, inositol phosphate; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin; Con A, concanavalin A; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; $Fc_\epsilon R$, FC receptor for immunoglobulin E; PAF, platelet-activating factor; IL-4, interleukin-4; IL-2, interleukin-2; ACTH, corticotropin; PKC, protein kinase C; and fMLP, formyl-methionyl-leucyl-phenylalanine.

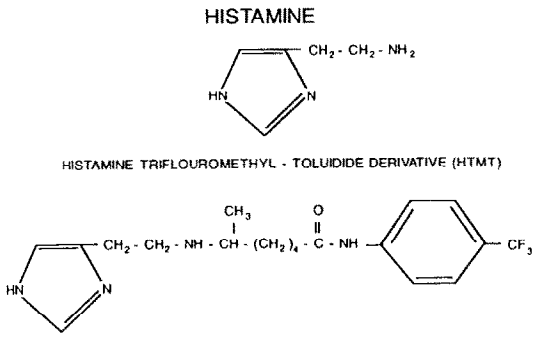

Fig. 1. Structures of histamine and histamine trifluoromethyl-toluidide derivative.

We have identified the HTMT receptors on non-rosetted cells, but the rosetted cell population does not respond to HTMT.

We have reported previously that the expression/function of the H_2 receptor is regulated by mitogens (PHA) [3]. Others also have reported that many lymphokines and pharmacologic agents modulate the expression of cell surface receptors [9–14]. For example, pretreatment of cloned helper T lymphocytes with IL-2 causes unresponsiveness of TCR to antigen and Con A. This is associated with decreased production of inositol phosphate and diacylglycerol [9]. IL-4 induces the expression of $Fc_\varepsilon R$ (Fc receptors for immunoglobulin E) and IL-2 receptors on murine B cells [10, 11], and transferrin receptor on T cells [12]. In addition, LPS and Con A increase the binding of ACTH in rat T and B lymphocytes [13], whereas LPS and PMA down-regulate the receptor expression for leukotriene B_4 [14].

In this study, we investigated how the response to HTMT could be regulated by IL-4, IL-2, LPS, and PMA, and have compared the effects of these agents with anti-IgM- and PAF-induced Ca^{2+} responses. We found that pretreatment with IL-4 and LPS increased the cellular response to HTMT in non-rosetted cells. The increased response of Ca^{2+} by IL-4 was inhibited when protein synthesis was inhibited but was not affected by inhibitors of RNA transcription. In contrast, the effects of LPS were not blocked by inhibitors of either protein synthesis or RNA transcription.

MATERIALS AND METHODS

Pharmacologic agents. The details of the synthesis and structure of HTMT have been described [6, 7]. The structure of HTMT is shown in Fig. 1. Human recombinant IL-4 was purchased from Genzyme Inc. (Cambridge, MA), and LPS was obtained from DIFCO Laboratories (Detroit, MI). Human recombinant IL-2 was a gift from the Cetus Corp. (Emryville, CA). PMA, PAF, cyclohexamide, and actinomycin D, were all obtained from the Sigma Chemical Co. (St. Louis, MO). Goat anti-human IgM was purchased from TAGO Immunologicals (Burlingame, CA).

Separation of leukocytes. PBL were isolated by Ficoll-Hypaque gradient centrifugation of fresh defibrinated blood. The subsets of lymphocytes (T and non-rosetted cells) were separated by a one-step rosetting method using neuraminidase-treated sheep erythrocytes (SRBC) [15]. The non-rosetted cells were suspended in complete medium (RPMI 1640, 10% fetal bovine serum, 2 mM glutamine, 100 μg/mL penicillin/streptomycin). Cells that had adhered to plastic dishes incubated overnight at 37° were removed. The resulting preparation contained 20–30% of CD19 and CD20 and 60–70% of CD14 positive cells.

Measurement of $[Ca^{2+}]_i$. Cells were treated with lymphokines or mitogens for 48 hr before measuring HTMT-induced calcium mobilization and IP production. Cells were washed with RPMI 1640 and loaded with 3 μM Indo-1 AM (Molecular Probes, Eugene, OR) for 30 min at 37°. Indo-1-loaded cells were then washed and resuspended in phosphate-buffered saline (PBS) containing Ca^{2+} and Mg^{2+} (GIBCO) supplemented with 10 mM HEPES, pH 7.4. Fluorescence intensity was measured by an SLM 8000 spectrofluorometer. Calcium concentrations were calculated according to the method of Rabinovitch *et al.* [16]. Data are expressed as the percentage of the response to a given concentration of the agonist.

Inositol phosphate production. Measurement of inositol phosphate has been described [8]. Briefly, cells were incubated with 10 mCi/mL of [^{3}H]-*myo*-inositol (14.6 Ci/mmol, NEN) at 37°. Then the labeled cells were washed three times with PBS, and the experiments were carried out in PBS containing 10 mM LiCl. The levels of inositol phosphate metabolites (IP_1, IP_2, IP_3) were determined by ion-exchange chromatography [17].

RESULTS

Pretreatment with IL-4 and the effects of HTMT. After non-rosetted cells were pretreated with IL-4 for 48 hr, there was no significant change in cell number and viability. The IL-4 (50–100 U/mL) by itself did not alter basal levels of $[Ca^{2+}]_i$ or increase $[Ca^{2+}]_i$. HTMT incubated with fresh cells elevated the $[Ca^{2+}]_i$ in a concentration-dependent manner, with an EC_{50} of 3.9×10^{-5} M. Cells treated with IL-4 and then with HTMT showed a left shift in the concentration–response curve with an EC_{50} of 2.0×10^{-5} M (Fig. 2). HTMT (3×10^{-5} M) in fresh cells increased $[Ca^{2+}]_i$ 95% over the basal levels (263 ± 12 mM), but in cells pretreated with IL-4 the increase in $[Ca^{2+}]_i$ was 204% (Fig. 3). Pretreatment with IL-4 ranged from 10 to 500 U/mL. Augmentation of responses to HTMT commenced at 50 U/mL and reached a peak of 200–400 U/mL (Fig. 4). The kinetics of augmentation of the effects of HTMT on $[Ca^{2+}]_i$ flux are shown in Fig. 5. After 12 hr of incubation with IL-4, the cells began to show augmented responses to HTMT; such modulation reached a level of 70% enhancement over the untreated cells. After 48 hr, IL-4 was able to enhance the HTMT response by 140%. Cells

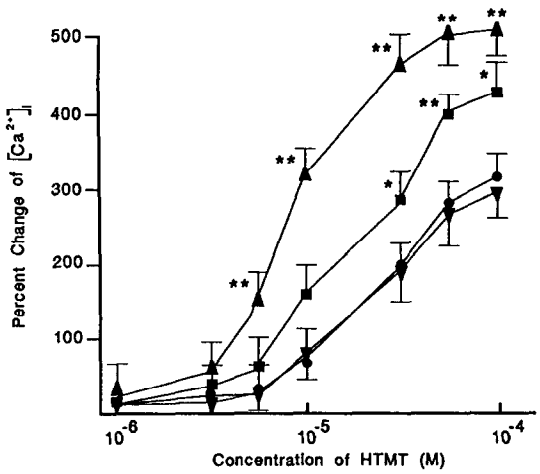

Fig. 2. Concentration–response relationship of HTMT on $[Ca^{2+}]_i$ in non-rosetted cells. Cells were incubated in the absence (▼) and the presence of 200 U/mL IL-4 (■), 50 μg/mL LPS (▲), and 60 μg/mL PMA (●) for 48 hr at 37°. Data are expressed as percent change over basal levels (263 ± 12 mM). Values are means ± SD from at least six independent experiments. Key: (**) $P < 0.01$, and (*) $P < 0.05$ compared with HTMT alone.

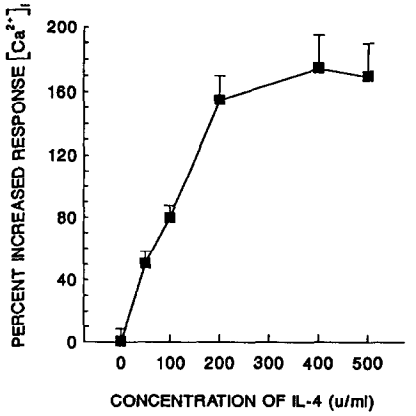

Fig. 4. Effects of IL-4 on HTMT-induced responses in $[Ca^{2+}]_i$. Cells were preincubated with different concentrations of IL-4 for 48 hr at 37°. The Ca^{2+} flux was then perturbed by HTMT (5×10^{-5} M). Data are expressed as percent increase in $[Ca^{2+}]_i$ after pretreatment with IL-4 compared with the responses in untreated cells (263 ± 12 mM). Each point is the mean ± SD from at least three separate experiments.

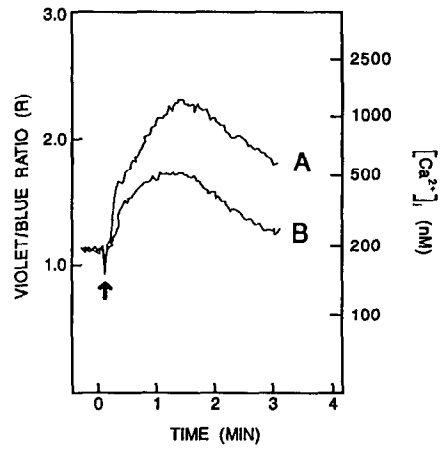

Fig. 3. Effect of IL-4 on HTMT-induced $[Ca^{2+}]_i$ elevation in non-rosetted cells. Ca^{2+} flux was induced by HTMT (3×10^{-5} M). Cells were incubated with IL-4 (200 U/mL) for 48 hr before $[Ca^{2+}]_i$ measurement. A representative experiment of changes in $[Ca^{2+}]_i$ in cells with (A) and without (B) IL-4 pretreatment is shown.

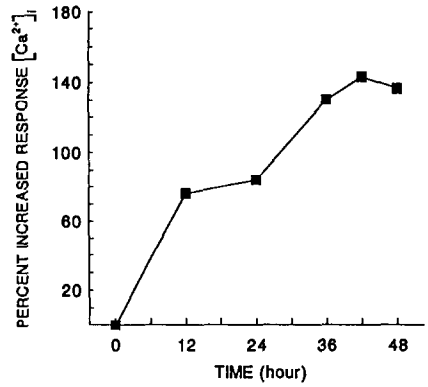

Fig. 5. Time-course of the augmentative effects of IL-4 on HTMT-induced responses of $[Ca^{2+}]_i$. Cells were pretreated with 200 U/mL of IL-4 for 12, 24, 36, 42, and 48 hr. HTMT (3×10^{-5} M) was added to induce Ca^{2+} mobilization. Data are the means ± range (within the symbol) of duplicates from a representative experiment.

treated with IL-4 (200 U/mL) for 48 hr were used for the rest of the experiments.

Effects of LPS and PMA on HTMT-induced Ca^{2+} *mobilization.* LPS has been shown to be a potent stimulant of calcium mobilization in human neutrophils induced by fMLP [18], whereas PMA could either inhibit or stimulate Ca^{2+} flux in both T or B lymphocytes activated by surface antibody or mitogens [19–22]. We have studied the effects of LPS and PMA on HTMT-induced $[Ca^{2+}]_i$ flux. Cells pretreated with LPS (50 μg/mL) or PMA (60 μg/mL) for 48 hr produced no changes in cell number or viability. As shown in Fig. 2, the increase in $[Ca^{2+}]_i$ induced by HTMT in LPS-pretreated cells was much higher than in the control cells. The EC_{50} shift from 3.9×10^{-5} M to 1.1×10^{-5} M was more profound than in cells pretreated with IL-4. On the other hand, incubation with PMA for 48 hr did not affect HTMT-induced $[Ca^{2+}]_i$ response. The EC_{50} value of the HTMT remained the same in pretreated and control cells (Fig. 2).

Effects of IL-4, PMA, and LPS on Ca^{2+}

Table 1. Effects of cytokines and mitogens on Ca^{2+} flux*

Treatment	% Change in $[Ca^{2+}]_i$		
	HTMT	PAF	Anti-IgM
Control	140.6 ± 16.9	691.1 ± 3	162.4 ± 17.3
IL-4	285.0 ± 18.6†	898.5 ± 129	168.0 ± 55.6
LPS	392.2 ± 90.7†	846.8 ± 66.4	129.7 ± 13.2
PMA	129.2 ± 10.4	660.2 ± 111	127.0 ± 28.3
IL-2	173.9 ± 8.4	542.5 ± 91	238.2 ± 98.7†

* Ca^{2+} mobilization was induced by HTMT (3×10^{-5} M), PAF (5 μg/mL), and anti-IgM (100 μg/mL). Cells were incubated with IL-4 (200 U/mL), LPS (50 μg/mL), PMA (60 μg/mL), or IL-2 (100 U/mL) for 48 hr before adding the agonists. Data are expressed as percent change over basal $[Ca^{2+}]_i$ level (263 ± 12 mM). Values are means ± SD from at least three independent experiments.

† $P < 0.05$ compared with control.

Table 2. Effects of the inhibitors of protein synthesis and RNA transcription on IL-4-mediated increase in response to HTMT*

Treatment	% Change in $[Ca^{2+}]_i$
Control	136.2 ± 15.4
IL-4	356.3 ± 34.0†
IL-4 + cyclohexamide	150.5 ± 53.5‡
IL-4 + actinomycin D	343.4 ± 79.8

* $[Ca^{2+}]_i$ mobilization was induced by HTMT (3×10^{-5} M). Cells were incubated with IL-4 (200 U/mL) or IL-4 plus cyclohexamide (1 μg/mL) or IL-4 plus actinomycin D (1 μg/mL) for 48 hr before adding HTMT. Data are expressed as the percent response over basal levels (263 ± 12 mM). Values are means ± SD from at least five independent experiments.

† $P < 0.05$ compared with control.

‡ $P < 0.05$ compared with IL-4 only.

mobilization induced by various pharmacologic agents. We further studied the effects of lymphokines (IL-2 and IL-4), PMA and LPS on Ca^{2+} flux. Cells were treated with these agents for 48 hr, and Ca^{2+} mobilization was induced by HTMT, PAF and anti-IgM. PAF increases $[Ca^{2+}]_i$ by activating specific cell-surface receptors [23], whereas anti-IgM stimulates the Ca^{2+} signal by cross-linkage of membrane IgM on B cells [24]. As shown in Table 1, elevation of $[Ca^{2+}]_i$ induced by HTMT was modulated by IL-4 and LPS as described before, but was not affected by IL-2 or PMA. PAF caused a 691.4% increase in $[Ca^{2+}]_i$ in untreated cells. However, 22–30% of that increase was observed after cells were treated with IL-4 and LPS. These differences were not statistically significant. Similarly, IL-2 and PMA did not affect the $[Ca^{2+}]_i$ response induced by PAF. On the other hand, none of the above-mentioned agents, i.e. IL-4, LPS, or PMA, had any effect on anti-IgM-induced $[Ca^{2+}]_i$ mobilization, indicating that the change in responsiveness in $[Ca^{2+}]_i$ was agonist specific. A 50% increase in response ($P < 0.05$) induced by anti-IgM was observed after cells were preincubated with IL-2 (100 U/mL).

Mechanisms of IL-4 and LPS effects on augmentation of HTMT-induced responses. To study the possible mechanisms of modulation of HTMT-induced Ca^{2+} response by IL-4 and LPS, we used a protein synthesis inhibitor, cyclohexamide, and an RNA transcription inhibitor, actinomycin D. Cells were incubated with IL-4 or LPS alone or in combination with cyclohexamide and/or actinomycin D. Cyclohexamide and actinomycin D at 1 μm/mL did not affect HTMT-induced Ca^{2+} responses by themselves. As shown in Table 2, the increased $[Ca^{2+}]_i$ induced by IL-4 was blocked by cyclohexamide but not by actinomycin D. On the other hand, the response to LPS was not altered by either cyclohexamide or actinomycin D (Table 3). These observations indicate that IL-4 and LPS modulated the HTMT-induced Ca^{2+} response through different mechanisms.

Modulation of HTMT-induced inositol phosphate production by IL-4 and LPS. We have proposed that IP is a possible second messenger for $[Ca^{2+}]_i$ elevation induced by HTMT through the HTMT receptors [8]. Thus, agents that modulated $[Ca^{2+}]_i$ should also affect IP formation. As predicted, IL-4 and LPS increased, but PMA had no effect, on the IP response induced by HTMT (Fig. 6). Furthermore, there was a good correlation between $[Ca^{2+}]_i$ increase and release of IP, implying that the IP changes were HTMT receptor-mediated responses.

Table 3. Effects of the inhibitors of protein synthesis and RNA transcription on HTMT-induced Ca^{2+} response in the presence of LPS*

Treatment	% Change in $[Ca^{2+}]_i$
Control	171.6 ± 31
LPS	597.7 ± 37†
LPS + cyclohexamide	598.0 ± 65
LPS + actinomycin D	518.6 ± 138

* Cells were incubated with LPS (50 μg/mL) or LPS plus cyclohexamide (1 μg/mL), or LPS plus actinomycin D (1 μg/mL) for 48 hr. Then HTMT (3×10^{-5} M) was used to induce Ca^{2+} mobilization. Data are expressed as percent change over basal $[Ca^{2+}]_i$ level (263 ± 12 mM). Values are means ± SD from at least five independent experiments.

† $P < 0.05$ compared with control.

DISCUSSION

In this study, we investigated the effects of lymphokines and mitogens on HTMT-induced $[Ca^{2+}]_i$ and IP responses in non-rosetted cells isolated from human PBL. The non-rosetted cell population contained 20–30% of B lymphocytes and 60–70% of monocytes. Further separation showed that monocytes responded to HTMT. We found that after pretreatment with IL-4 and LPS, the HTMT-induced $[Ca^{2+}]_i$ flux and IP accumulation were augmented in these cells. In contrast, PMA and IL-2 did not have any modulatory effect on HTMT-

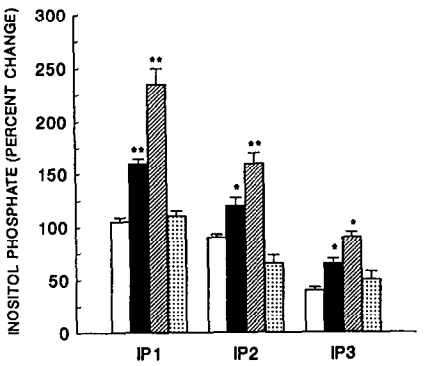

Fig. 6. Effects of IL-4, LPS and PMA on inositol phosphate formation induced by HTMT. Cells were incubated with HBSS (open bars), IL-4 (200 U/mL) (solid bars), LPS (50 μg/mL) (striped bars), and PMA (60 μg/mL) (dotted bars) for 48 hr. Then inositol phosphate production was studied in the presence of HTMT (5×10^{-5} M). Data are the means ± SD of triplicates from a representative experiment. Key: (*) $P < 0.01$, and (**) $P < 0.05$ compared with untreated cells (cells in Hanks' balanced salt solution).

induced responses. In our previous studies, we showed that the effects of histamine congeners on $[Ca^{2+}]_i$ and IP responses were mediated solely via a newly discovered HTMT receptor that was different from the H_1, H_2, or H_3 receptors [8].

When non-rosetted cells were pretreated with IL-4, there was a markedly enhanced response to HTMT-induced Ca^{2+} flux and IP formation. The effect of IL-4 on the HTMT-induced response seemed to be agonist specific. The Ca^{2+} response induced by PAF was not affected by IL-4. In addition, this phenomenon was also lymphokine specific, since IL-2 did not affect HTMT-induced responses. It has been suggested that IL-4 indirectly affects B cell function by preparing these cells to respond more promptly to subsequent stimulation [25]. For example, the IL-4-treated B cells enter S phase much faster than untreated cells when stimulated with anti-IgM or PMA. Interestingly, although the effects of anti-IgM that stimulate resting B cells to enter S phase are mediated by increasing $[Ca^{2+}]_i$ and IP metabolism, B cells pretreated with IL-4 did not respond to anti-IgM by enhanced accumulation of IP or greater mobilization of $[Ca^{2+}]_i$. Nor do IL-4-treated B cells show any increase in protein kinase C activity when stimulated with PMA [26]. Similarly, IL-4 enhances HLA-DR expression on human monocytes and this effect does not seem to be mediated by Ca^{2+}/PKC signalling [27]. In the present study, we also demonstrated that IL-4 did not modulate the $[Ca^{2+}]_i$ response induced by anti-IgM in which the responding population was the B cells in the non-rosetted population. However, the increase in $[Ca^{2+}]_i$ and the production of IP resulting from activation of HTMT receptors on monocytes were augmented after cells were treated with IL-4.

The mechanisms of IL-4 effects on HTMT-mediated responses are not clear. The augmentation of response was not due to the increased cell number since IL-4 affected neither the cell count nor viability. The requirement of prolonged incubation with IL-4 to modulate the HTMT response implies that some cellular signal is required over an extended period of time. We found that the effects of IL-4 were not reversed by an inhibitor of RNA transcription, but were blocked by an inhibitor of protein synthesis. It has been reported that IL-4 suppresses RNA levels in human monocytes [28, 29]. Oliver *et al.* [30] reported that IL-4 does not increase RNA content in resting B cells sufficiently to be scored as having entered the G_{1A} phase of the cell cycle, indicating that IL-4 action does not require any changes in RNA content. It has been suggested that IL-4 induces autocrine production of a B cell growth factor, thereby affecting B cell function [25]. Whether IL-4 directly modifies biosynthesis or turnover of HTMT receptors or requires release of unidentified B cell growth factor which then, in turn, affects Ca^{2+} response in monocytes remains to be elucidated.

In our studies, LPS was more potent than IL-4 in inducing the HTMT-mediated $[Ca^{2+}]_i$ and IP responses. However, LPS did not affect the Ca^{2+} response induced by PAF as suggested by others [31]. The effects of LPS were not blocked by inhibitors of either protein synthesis or RNA transcription. These results suggest that LPS did not modify the HTMT-mediated responses by the same mechanism(s) as IL-4, since cyclohexamide inhibited IL-4-mediated actions on HTMT receptors. LPS is known to increase RNA synthesis in both human monocytes and mouse B cells [32, 33]. The negative effects of RNA transcription inhibitor on LPS-induced changes in $[Ca^{2+}]_i$ and IP responses in human non-rosetted cells indicated that LPS did not affect the RNA content. LPS has been suggested to act directly through activating protein kinase C [34]. It is unlikely that LPS acted through protein kinase C in our system, since PMA, a protein kinase C activator, did not affect HTMT-mediated Ca^{2+} and IP responses. Other investigators also have shown that LPS enhances Ca^{2+} response induced by fMLP in human neutrophils [18]. Furthermore, LPS can either up-regulate (ACTH) or down-regulate (LTB_4) the expression of cell surface receptors [13, 14]. The mechanisms of such actions by LPS have not been determined.

PMA was used to further compare the effects of IL-4 and LPS on Ca^{2+} mobilization. Cells treated with PMA for 48 hr did not exhibit any changes in Ca^{2+} response in our system. On the other hand, it has been reported that in Jurkat and B cells short-term treatment (less than 2 hr) with PMA inhibits elevation of both Ca^{2+} and IP responses by a negative feedback mechanism [19–21]. However, long-term treatment (over 24 hr) with PMA usually results in an enhanced response because PKC is depleted from the cell membrane [19, 35, 36], and the feedback inhibition is abolished. Furthermore, in normal human T cells, short-term treatment with PMA markedly enhances Ca^{2+} response and the stimulatory response of PMA starts to diminish after cells are incubated with PMA for 6 hr [21, 22, 37]. Nevertheless, it is not clear how the non-rosetted cells in our experiments responded to the short-term

treatment with PMA since there was no change in Ca^{2+}/IP response after long-term PMA incubation. It is unlikely that PMA affected the cell surface expression of HTMT receptors, although it has been shown to regulate fMLP receptor levels in human neutrophils [38].

In summary, we report that the HTMT-mediated responses in human non-rosetted cells can be modulated by IL-4 and LPS. Such interactions may be factors in histamine-induced immune modulation.

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