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C-Kit Ligand Promotes Mast Cell Infection by *Toxoplasma gondii*

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Abstract: Biological functions of mast cells include a functional role in innate immunity against parasitic infections. Here, we demonstrated that mast cells could also play a role in the anti-microbial defenses regulation and might participate as a parasite reservoir. We observed that *Toxoplasma gondii* infected massively *in vitro* mouse bone marrow derived mast cells (BMMC), a mucosal mast cell (MMC) phenotype, followed by substantial cell lysis. This induced release of β-hexosaminidase, but not of preformed or neosynthesized TNF-α. Culturing MMC in the presence of recombinant mouse stem cell factor (c-kit ligand) led to their maturation into connective tissue-like mast cells (CTMC), which *T. gondii* was able to adhere on and to infect more. *T. gondii* infection did not induce release of β-hexosaminidase and serotonin from BMMC. These results demonstrated that mast cells interact with *T. gondii* and are massively infected, especially after their maturation by c-kit ligand.

Keywords: Mast cells, *Toxoplasma gondii*, C-kit, TNF-α, β-hexosaminidase.

INTRODUCTION

Cytokines derived from macrophages and T cells are known to play an important regulatory role in host defense and to control the susceptibility to microbial infection. IL-1-α, IL-6, IFN-γ, IL-12 and TNF-α are particularly involved in host protection against *Toxoplasma gondii* infection [1]. During *T. gondii* infection an important role for IFN-γand TNF-α has been proposed both in the development of the disease and the mobilization of efficient defense mechanisms [2-5]. *Toxoplasma* is an obligate intracellular parasite. Its attachment to the host cell is the first step required for the mechanism of invasion, and the second step is critical for its silent survival. Unfortunately, the process for the identification of parasite ligands and cell surface receptors remains incomplete [6, 7].

Mast cell is the only cell type that stocks preformed TNF-α in their granules [8]. The rapid release of TNF-α is crucial during *T. gondii* infection since it can exert a direct cytotoxic effect on microorganisms and an indirect effect by recruitment of phagocytic cells at the sites of infection [5]. Mast cells are found in relatively large number of adjacent to blood or lymphatic vessels but are most prominent immediately beneath the epithelial surface of the skin and the mucosa of the genitourinary, gastrointestinal, and respiratory tracts [9-11]. Moreover, mast cells release a large number of preformed mediators and produce neosynthezised mediators against external stimuli. In addition to inflammatory factors such as biogenic amines, leukotrienes and prostaglandins, mast cells produce and release a large panel of cytokines such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IFN-γ, TNF-α, GM-CSF, MIP-1α, MIP-1α and TGF-β [9, 12]. There is considerable evidence that mast cells recognize and react to a wide range of microorganisms or their products.

Stem cell factor (SCF), also termed Kit ligand, steel factor or mast cell growth factor is the ligand of the *c-kit* protooncogene product [13]. Therefore, SCF has been first described as a pluripotent growth factor involved in the early stages of haematopoiesis. Recently, it has been described to be also implicated in inflammatory processes [14].

Infection with *T. gondii* is acquired through the oral route by ingestion of undercooked or raw meat containing cysts of the parasite or through ingestion of water or food contaminated with cysts or oocysts. Following ingestion, sporozoites are released from cysts and rapidly invade the intestinal mucosa and convert into tachyzoites.

These observations suggested that mast cells may represent one of the first inflammatory cells encountered by an invading pathogen. Mast cells can exert cytotoxic activity against various pathogens, such as *Schistosoma mansonii* [15], *Leishmania parasites* [16,17], *Escherichia coli* [18] and

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Klebsiella pneumoniae [19], but their role against *T. gondii* infection remains poorly studied.

For this reason, we first studied the interaction between the mucosal mast cells (MMC) and *T. gondii* parasites and we analysed: 1) the possibility of MMC to phagocytose *T. gondii,* and 2) the role of preformed mediators (biogenic amines, TNF-α) during *in vitro* co-incubation. We also studied the interaction between MMC after their maturation into connective tissue-like cells (CTMC) and *T. gondii.*

MATERIALS AND METHODOLOGY

Mast Cell Cultures

Bone marrow-derived mast cells (BMMC) were obtained from femurs of 2-months-old male BALB/c mice (IFFA-CREDO, L'arbresle, France) and were cultured at 37°C in a humidified atmosphere containing 5% CO_2 at a starting density of 3.10^5 cells/ml in RPMI-1640 medium supplemented with L-glutamine, penicillin, streptomycin, 10% FCS (complet medium) (all from Gibco Laboratories) and 2 ng/ml murine recombinant IL-3 (rIL-3; Immugenex, Los Angeles, CA). The medium was renewed every 5 to 7 days. After 28 days in culture, a sample of the cell suspension was stained with toluidine blue solution (0,1% in 50% ethanol; pH 3,5). More than 98 % of the cells used were identified as mast cells by the presence of metachromatic granules. These cell cultures presented the mucosal phenotype and were then used for subsequent experiments as pure mucosal mast cell populations. Mast cells obtained after a 4-weeks culture period in the presence of r-IL-3 were further cultured for 14 days in the presence of 40 ng/ml mouse recombinant c-kit ligand (Amgen, Thousand Oaks, CA). The differentiation into connective tissue mast cells and the mast cell phenotype were monitored by toluidine blue and alcian blue-safranine (ABS) staining [20, 21].

Toxoplasma gondii Isolation

Virulent *T. gondii* RH strain, maintained in Balb/c mice by intraperitoneal passage was isolated by peritoneal lavage following filtration. The RH strain never induces cystic forms in mice and kill mouse after 3 to 4 days. Tachyzoites were used in our studies and their viability was controled by trypan blue dye exclusion and only preparations with > 95% viable parasite were used. In our experiments Balb/c mice (IFFA-CREDO) were used at 6 to 8 weeks of age. During experimental protocols, all mice were maintained in specific-pathogen-free facilities.

Co-Culture of BMMC with *T. gondii* Parasites

To determine the infectivity and the interactions between mast cells and *T. gondii* parasites, BMMC cultures (10^6cells/ml) were exposed to *T. gondii* tachyzoites at various ratios of parasites per cell and incubated in 24-multiwell culture plates with the complet medium at 37° C in humidified air, containing 5 % CO2. After various periods, the co-cultures were examined by direct parasitological studies of May-Grunwald Giemsa (MGG) stained cytospin preparations. The level of direct interactions between mast cells and *T. gondii* and the percentage of intracellular infection was evaluated microscopically by examining over 500 mast cells in three separate experiments.

Electron Microscopy

For morphologic studies by transmission electron microscopy (TEM), pellets of infected mast cells at different periods of culture were fixed in 2% glutaraldehyde and 0.1 M sodium cacodylate buffer, at PH 7.4. After glutaraldehyde fixation, the samples were processed for transmission electron microscopy.

Mediators Release by Mast Cells

For the measurement of β-hexosaminidase and TNF-α release, BMMC cultures (10^6cells/ml) were exposed (in 24-multiwell culture plates) to *T. gondii* tachyzoites at a ratio of 1 and 4 parasites per cell and incubated in white RPMI 1640 (without red phenol) plus 1 % bovine serum albumin (BSA). The reaction mixtures were centrifuged at 500g for 10 min at 4°C. The supernatants and pellets were collected at different time of coculture and stored at – 80°C until assay. In addition, to determine maximal mediator release ability of BMMC after IgE-immune complex stimulation, cells (one million per ml in RPMI-1640 devoid of phenol red) were optimally sensitised by incubation at 37°C for 60 min with 10 µg/ml of mouse monoclonal IgE directed against Dinitrophenyl (DNP; ICN Biomedicals, Costa Mesa, CA). The cells were then washed twice, and resuspended at 10^6 cells/ml in the same medium, then seeded in 24-well plates (1ml per well). At this time, 10 ng of DNP-BSA (Calbiochem, La Jolla, CA) were added in each well for 60 min.

β-Hexosaminidase Assay

β-hexosaminidase release was measured after coculture of BMMC with *T. gondii* at 37°C as described above. Briefly, β-hexosaminidase was measured in the supernatants and extracts of sonicated mast cell sediments after 40 min, 4, 24 and 48 hours of culture by hydrolysis of p-nitrophenyl-2-acetamido-2-deoxy-β-D glucopyranoside exactly as described elsewhere [22, 23]. The percentage of release of β-hexosaminidase was calculated by the following formula: net percent release = $[S - S^{control}]/[(S + P) - S^{control}] \times 100$, where S is the mediator content of supernatant cells, P is the mediator content of pellet of stimulated cells, and $S^{control}$ is the mediator content of supernatant of unstimulated cells.

Serotonin Assay

Cell supernatants (250 µl aliquots) were supplemented with 5 % (v/v) $HClO_4$, 0.05 % (w/v) ($Na_2S_2O_5$ and 0.05 % (w/v) disodium ethylene diaminetetraacetic acid (EDTA), and the resulting precipitate was removed by centrifugation at 30000 g for 15 min at 4°C. Clear supernatants were then neutralized by the addition 30 µl of 2 M KH_2PO_4/K_2HPO_4, pH 7.4 and supplemented with ascorbate oxidase (0.01 mg/ml, Boehring Manuhein Germany), before a second centrifugation as above to remove $KclO_4$ precipitate. Aliquots (10 µl) of resulting clear supernatants were injected directly into a high-performance liquid chromatography (HPLC) column (Ultrasphere IP, Beckman, Gagny, France; 25 x 0.46cm, C18 reversed-phase, particle size 5 µm) protected with a brownlee precolumn (3cm, 5µm). The mobile phase (at a flow rate of 1 ml/min) consisted of (in mM): KH_2PO_4, 70; triethylamine, EDTA, 0.1; octane sulphonate, 1.25, and 16 % methanol, adjusted to pH 3.02 with solid citric acid [24]. Serotonin (5-hydroxytryptamine, 5 HT) eluted from the

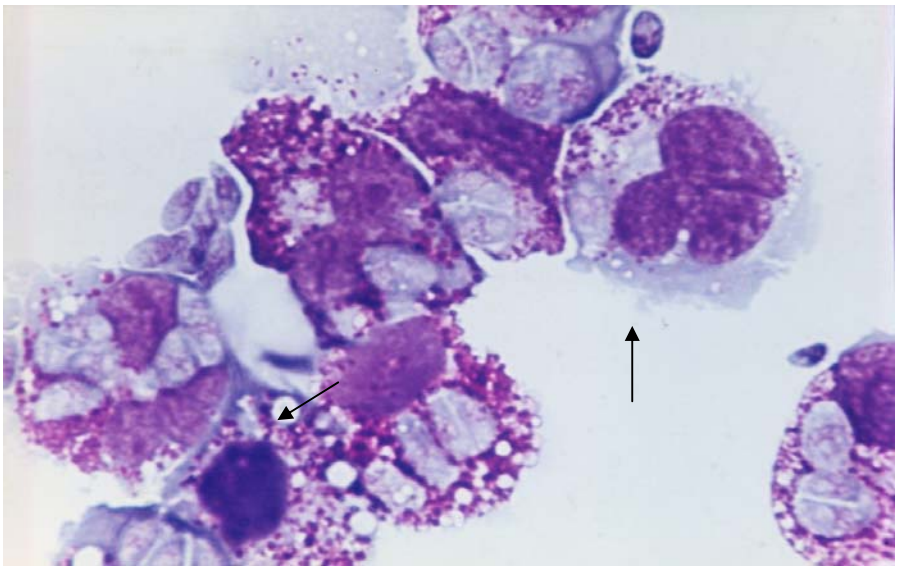

Fig. (1). Direct interactions between *T. gondii* and mast cells.
Co-cultures of BMMC (10^6 cells) with *T. gondii* (10^6parasites) at 37°C-5% CO_2 show clear binding of multiple parasites on mast cell membrane (May-Grumwald Giesmsa staining, x 300). Intracellular *T. gondii* parasites (arrow) obtained after 1h of coculture with BMMC.

column (retention time = 32 min) was monitored by an electrochemical detection system (ESA 5011, Bedford, USA) made of an analytical cell with dual coulometric monitoring electrodes (+ 50 mV and + 350 mV). The generated signal was integrated by a computing integrator (Millenium^{32}– Waters, Saint-Quentin-en-Yvelines, France). Quantitative determination of 5-HT was made by reference to appropriate external standards (10 pg – 10 ng) authentic 5 HT, (Sigma, Saint-Quentin Fallavia, France).

TNF-α Assay

TNF-α production was measured in the cell supernatants after 1, 3 and 6 hours of culture by a specific ELISA method (Mouse TNF-α ELISA Kit, Genzyme, Cambridge, MA). Results are expressed in pg/ml and the detection limit of the assay is 15pg/ml.

Statistical Analysis

Data are reported as mean ± SD of separate experiments. Differences were analyzed for significance ($p < 0.05$) by Student's t test.

RESULTS

T. gondii Infection of Mast Cells

Rapidly, in less than 30 minutes, *T. gondii* parasites adhered to cultured mast cells**.** The percentage of BMMC bearing parasites on their membrane and the number of parasites bound on these cells increased with parasite cell ratio. Indeed, after one hour of coculture at a mast cell-parasite ratio of 1:2, more than 40 % of the cells exhibited membrane bound *T. gondii* (4±3 parasites per cell). Following this interaction, the parasite penetrates the cell through their microvilli and replicates in the cytoplasm (Fig. **1**).

The percentage of mast cells infected by *T. gondii* (assessed by MGG staining) was 5±1 % and 8±2 % respectively for a ratio 1:1 and 1:2 after 1 hour of coculture. These percentages increased rapidely over one hour reaching, 16±6 and 30±9 after 4h, 85±5 and 94±4 at 24 hours (respectively for the ratios 1:1 and 1:2). The percentage of infection and the average numbers of parasites per infected cell were increased significantly with the time of the coculture (Table **1**).

Table 1. ***T. gondii in vitro*** **infection of BMMC at different time points. In this table, we report the rate (%) of infected mast cells and the number of tachyzoites per infected cell. Results represent the mean±SD of 500 cells, in three separate experiments**

Culture conditions	% of infected cells			Number of tachyzoites per infected cell		
	1h	4h	24h	1 h	4 h	24 h
BMMC (IL-3) + *T. gondii* (ratio 1:1)	5±1	16±6	85±5	1±1	3±1	7±2
BMMC (IL-3) + *T. gondii* (ratio 1:2)	8±2	30±9	94±4	1±1	5±2	10±3

The adherence of parasites on mast cells and the number of mast cells infected were more important with long-term treatment of MMC with SCF, allowing them to differentiate into CTMC. The percentage of CTMC and MMC infected at 5 hours is respectively 18 ± 7 % and 27 ± 8 % with the ratio (1:1) and respectively 42 ±10 % and 56 ±12 % with the ratio (1:4) (Table **2**).

Table 2. ***In vitro* interaction between 2 sub-types of mast cells and *T. gondii* parasites after 5 hours of coculture. In this table, we report the rate (%) of infected mast cells. Results represent the mean±SD of 500 cells, in three separate experiments**

Culture conditions	% of infected cells 5 hours
CTMC (IL-3) + *T. gondii* (ratio 1:1)	18±7
MMC (IL-3 + c-kit ligand) + *T. gondii* (ratio 1:1)	27±8
CTMC (IL-3)° + *T. gondii* (ratio 1:4)	42±10
MMC (IL-3 + c-kit ligand)° + *T. gondii* (ratio 1:4)	56±12

°Values significantly different from non stimulated with c-kit ligands BMMC ($p<0,05$).

The presence of parasites in the cytoplasm of mucosal mast cells was confirmed by transmission electron microscopy. The light microscopic examination showed areas of lysed cells, and the higher level of lysis was approximately obtained at 48 hours of co-culture with 10^6 mast cells and 10^6 parasites (date not shown). These data clearly demonstrate that all along the infectious process with *Toxoplasma* represent a putative important reservoir for parasites.

Ultra-Structural Examination

Rapidly, 10 minutes after co-culture, the tachyzoites adhered to the surface microvilli projections of the mucosal mast cells, and penetrated cells at apical site at approximately 30 minutes (Fig. **2a**). After 6 hours, *T.gondii* replicated into mast cells (Fig. **2b**), and the parasites occupied nearly all the cytoplasma and lysed in mast cells after 24 hours. Mast cell-induced *T. gondii* cytotoxic changes included a marked disruption of the surface membrane structures of the organisms, intracellular vacuolation were observed after 10 hours of coculture (Fig. **2c**).

Release of β-Hexosaminidase

The cell-membrane attachement of the parasite in mast cells induced a low release of β-hexosaminidase by BMMC after 60 minutes of culture (Fig. **3**). The maturation of MMC into CTMC by c-kit ligand did not enhance the release of β-hexosaminidase (data not shown). Even though the level of β-hexosaminidase release was lower to that observed after triggering the high affinity receptor of IgE (FcεRI), this time-dependent release of β-hexosaminidase is relevant. The release kinetic was similar to the lysis kinetic of the mast cells by *T. gondii* (Fig. **4**). In addition, mast cells/*T. gondii* parasites interaction did not interfere with the release of β-hexosaminidase after ligation of FcεRI receptors on these cells (Fig. **3**).

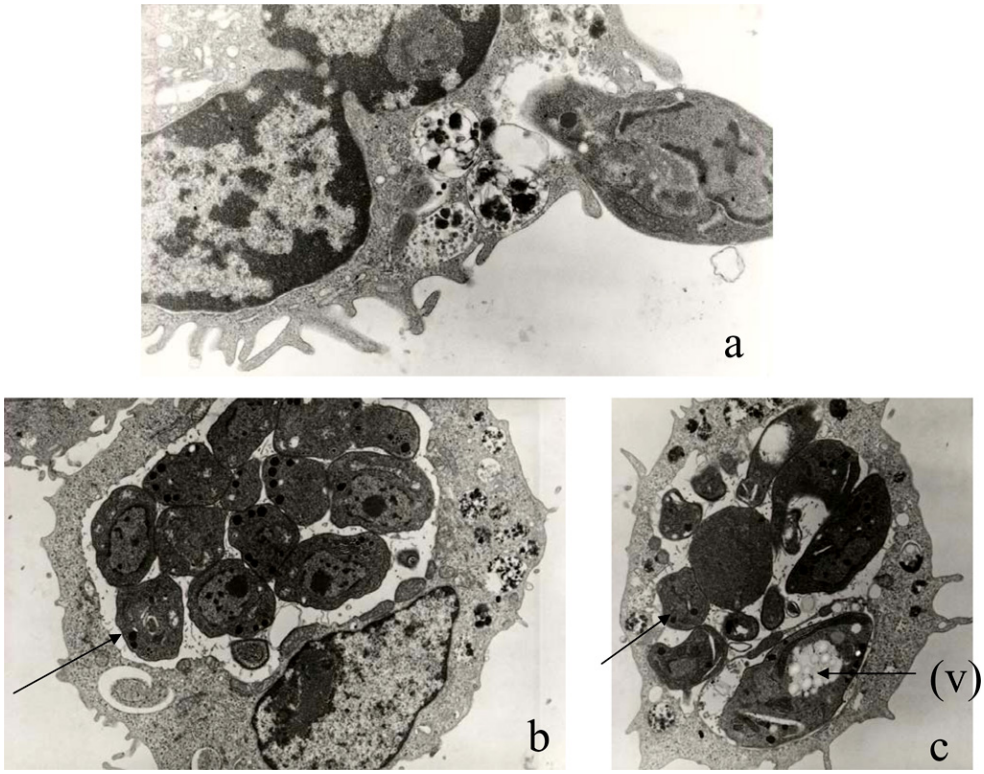

Fig. (2). Transmission electron microscopy examination of cross-section of BMMC showing: (a) the adherence of *T. gondii* to mast cell, (b) the internalization of the parasites and their proliferation, (c) mast cell-induced intracellular parasite cytotoxicity and vacuole formation in *T. gondii* are observed (V).

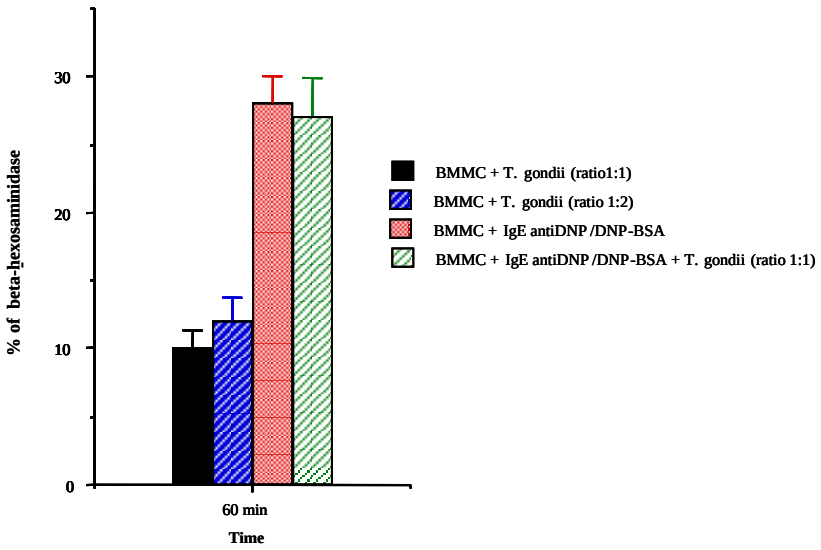

Fig. (3). Low-level degranulation of mast cells exposed *in vitro* to *T. gondii*.

The degranulation is appreciated by a β-hexosaminidase released by mast cells after 60 min and compared to maximal release obtained with optimal IgE antiDNP (10 µg/ml)/DNP-BSA (10 ng/ml), stimulation. Results represent the mean ± SD of three separate experiments.

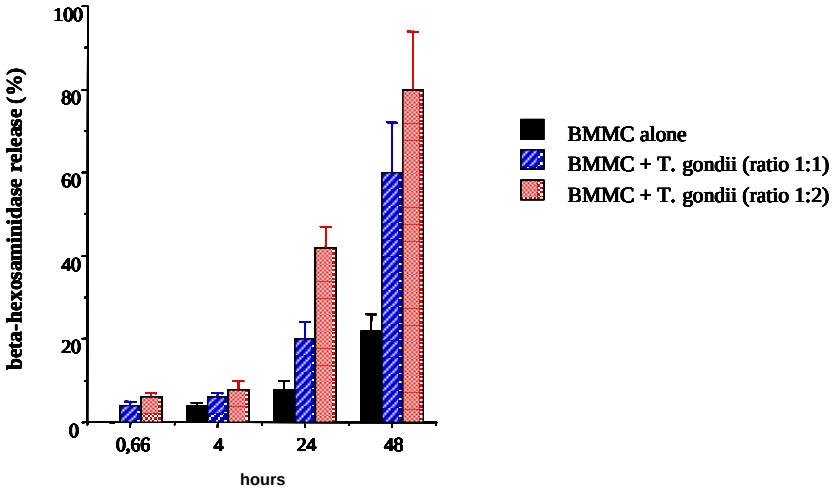

Fig. (4). Time-course released of β-hexosaminidase by BMMC (10^6 cells) exposed *in vitro* to *T. gondii* parasites at different ratio.

Release of Serotonin (5-HT)

Concomitantly to the release of β-hexosaminidase parasite/mast cell interaction did not induce the release of serotonin after 3 hours of co-culture**.** The maturation of MMC into CTMC by c-kit ligand did not enhance the release of serotonine by mast cells coculture with *Toxoplasma* parasites (Fig. **5**). However, the treatment by c-kit ligand enhanced the intracellular amount of serotonin by these cells (1070ng/10^6 with CTMC cells compared to 610ng/10^6 with MMC cells).

Release of TNF-α

Mast cells synthesize, store in granules and release continually a low amount of TNF-α and thus control the local immune homeostasis. We evaluated the effect of *T. gondii* binding to mast cells on this production. The coculture of BMMC with *T. gondii* parasites (ratio 1:1 and 1:2) did not induce a significant increase in secretion of TNF-α by these cells at 1 h, 3h and 6h (IgE anti DNP/DNP-BSA), compared to the amount of TNF-α constitutively released by mast cells at 24h (Fig. **6**).

DISCUSSION

The precise mechanisms by which mast cells could play a role during host invasion by *Toxoplasma* parasites are yet unknown. Recent data have evidenced a new role for these cells in innate immunity against bacteria and protozoan parasites, through their ability to be stimulated by their FcεRI or directly by these infectious agents [25-27]. Upon activation,

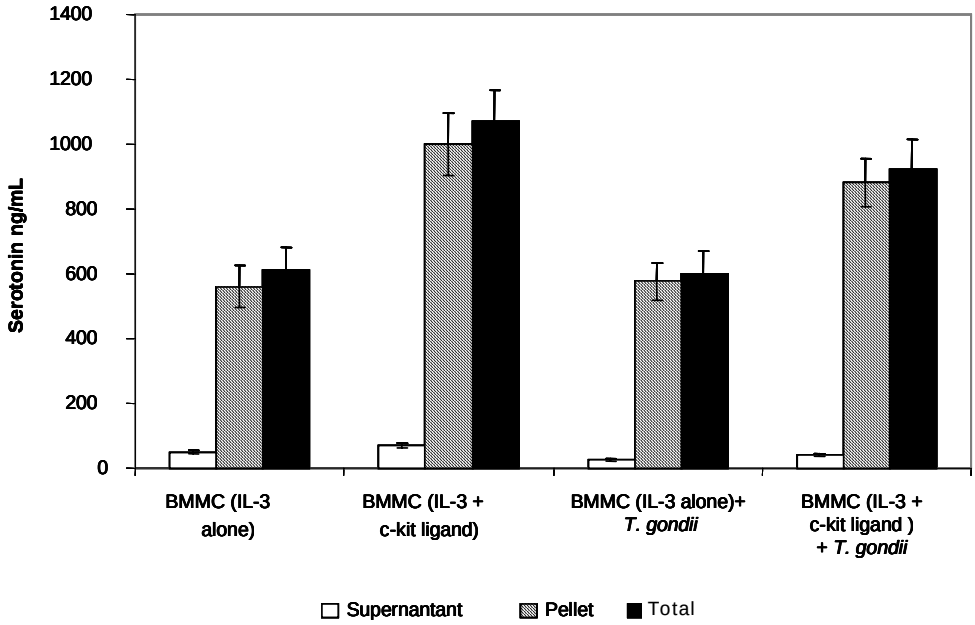

Fig. (5). The release of serotonin (5-HT) by two sub-types of (10^6) mast cells in co-culture with *T. gondii* (10^6) parasites after 3 hours, measured in supernatants and in the lysate pellet of cell preparations. Results represent the mean±SD of three separate experiments.

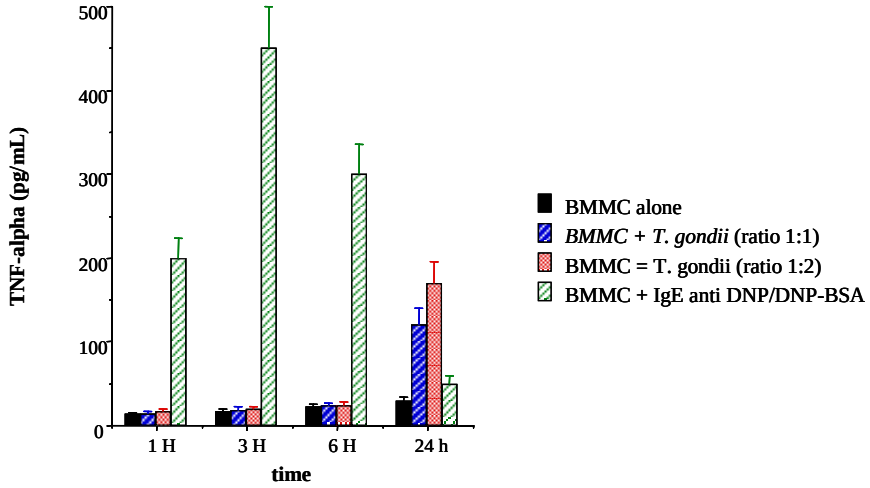

Fig. (6). Time-dependent release of TNF-α protein released by mast cells following *in vitro* exposure to *T. gondii* parasites as compared to that obtained after IgE/antigen stimulation. Results represent the mean ± SD of three separate experiments in the culture supernatants of BMMC.

mast cells release, within minutes, preformed mediators stocked in granules such as histamine, β-hexosaminidase and TNF-α. In addition, they are able to synthesize various cytokines, which account for long-term maintenance of the inflammatory response during allergic reactions [28, 29] and can take part in acquired immune responses against microorganisms [25-27].

We have previously demonstrated that *Leishmania* promastigotes adhere to mast cells and that a few parasites were phagocytosed and transformed into amastigotes by these cells [16], whereas, *T. gondii* invades these cells by an active process [30]. Moreover, *Leishmania*, an obligate intracellular parasite, can infect BMMC and induce the release of preformed mediators such as β-hexosaminidase and TNF-α. In addition, this interaction induces the neosynthesis of TNF-α by BMMC [16]. In contrast, data from the present study, clearly demonstrate that another obligate intracellular parasite, *T. gondii*, induces a low release of β-hexosaminidase and does not induce a significant release of serotonin and TNF-α from BMMC. Moreover, Pelloux and coworkers [31] have shown that *T. gondii* infection does not induce a sig-

nificant release of TNF-α from a human astrocytoma cell line after 1, 3, 6 and 24 hours of co-culture; although this cell line secretes constitutively TNF-α. Nevertheless, tachyzoites does not inhibit the secretion of TNF-α by human astrocytoma cell line activated by phorbol ester (PMA).

Henderson and coworkers [32] have shown that rat peritoneal mast cell-*T. gondii* interaction induced the release of histamine (preformed mediator stocked in granules as same at β-hexosaminidase) by these cells. These authors have co-cultured mature *in vivo* serosal mast cells and *T. gondii* with a higher ratio of parasites (1:6). To mimic this model, we have differentiated the *in vitro* MMC into CTMC by addition of c-kit ligand for 14 days. This maturation is objectived by the increase of the amount of serotonin (5-HT) in these cells (1070 ng/10^6 with CTMC compared to 610ng/10^6 with MMC). In our hands the interaction between CTMC and *T. gondii* did not enhance the release of β-hexosaminidase, but we have observed an increase of the parasite adherence on CTMC and of the number of infected cells, compared with MMC. In addition, the increase of the ratio parasite-mast cells does not enhance the release of β-hexosaminidase and serotonin (data not shown).

The discrepancies interactions between *Leishmania* and *Toxoplasma* towards mast cells are probably due to the difference of antigenic membrane profile on these two protozoans. Promastigotes of *Leishmania* species possess a major surface antigen, the glycoprotein gp63 kDa [33]. This major antigen is involved in macrophage binding, and can bind the fibronectin [34]. Similarily the major surface antigen of *T. gondii* (SAG-1 or P30) seems to be implicated in the adherence and invasion of various cells [35, 36]. Fibronectin and laminin could be implicated in the adherence of *Leishmania* promastigotes to cells and laminin, but not fibronectin could be implicated in the invasion of cells by *T. gondii*. [37].

Mast cells express many membrane receptors, a large number of receptors for cytokines, immunoglobulins and complement receptors. IL-3-dependent murine BMMC could only adhere to laminin or fibronectin after activation with PMA or after FcεRI aggregation, moreover, BMMC spontaneously adhered to vitronectin [38]. C-kit ligand factor was found to promote the adhesion of IL-3-dependent BMMC to fibronectin and lamilin [39-41].

We have observed that the percentage of infected mast cells with toxoplasma *parasites* increased and correlated with the number of parasites initially added to cells. The parasite replicated rapidly in mast cells, occupied the whole volume of these cells and lysed them. So, these cells release the whole amount of preformed TNF-α at 24hours (Fig. **6**), whereas serotinin remains only detectable in the mast cell pellets (data not shown). The entirety of infected mast cells was lysed between 24 and 48 hours of co-culture, and all of *T. gondii* parasites remaining extracellular.

A possible mechanism for how *T. gondii* parasites trigger mast cell degranulation may be through the release of lysophospholipids from the tachyzoites apical organelles, termed rhoptries [42]. Indeed, lysophospholipids, specially phosphatidylserine, potentialise mast cell histamine secretion [43]. Moreover, lysophosphatidylserine specifically activates connective mast cells *in vitro* [44, 45] and *in vivo* but not mucosal mast cells [46]. Have shown that *T. gondii* induces the release of histamine and synthesis of LTB4 by rat peritoneal mast cells an *in vivo* "serosal mast cell" model. On the contrary we have observed a low release of β-hexosaminidase and poor low release of TNF-α and serotonin by a "mucosal mast cell" model, and the differentiation of these cells into serosal mast cells *in vitro*, did not enhance the release of β-hexosaminidase and serotonin. In addition, these cells serve as a major chemokines (CXCR2) and cytokines (TNF-α) source involved in neutrophil recruitment [47] in the site of infection by *T. gondii* [48], and participate in the defense against microorganisms and protozoan parasites.

CONCLUSION

In this work, we demonstrated clearly that mast cells interact with *T. gondii* and that this interaction induces rapidly a high rate of infection and progressively lysis of mast cells. Finally, the increased adherence and infectivity of mast cells by *T. gondii*, is probably due to the expression of β integrins on mast cells after their treatment by c-kit ligand. Such investigations about the interaction between these two elements are now currently in progress in our laboratory.

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