IL-1 Induces Vesicular Secretion of IL-6 without Degranulation from Human Mast Cells[1]

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FcεRI cross-linkage in mast cells results in release of granule-associated mediators, such as histamine and proteases, as well as the production of numerous cytokines, including IL-6. Mast cells have been increasingly implicated in inflammatory processes where explosive degranulation is not commonly observed. Here, we show that IL-1 stimulates secretion of IL-6 without release of the granule-associated protease tryptase in normal human umbilical cord blood-derived mast cells (hCBMCs). IL-6 secretion stimulated by IL-1 in hCBMCs is potentiated by priming with IL-4 and reflects the higher levels of IL-6 secreted from human leukemic mast cell line (HMC-1). Stimulating HMC-1 cells by both IL-1 and TNF-α results in synergistic secretion of IL-6. IL-6 is de novo synthesized, as its secretion is blocked by inhibitors of transcription or protein synthesis. IL-1 does not increase intracellular calcium ion levels in either hCBMCs or HMC-1 cells, and IL-6 stimulation proceeds in the absence of extracellular calcium ions. Ultrastructural Immunogold localization shows that IL-6 is excluded from the secretory granules and is compartmentalized in 40- to 80-nm vesicular structures. Selective secretion of IL-6 from mast cells appears distinct from degranulation and may contribute to the development of inflammation, where the importance of IL-6 has been recognized. *The Journal of Immunology,* 2003, 171: 4830–4836.

Mast cells derive from a committed precursor in the bone marrow (1) and enter the tissues where they develop specific phenotypes in response to local stimuli, such as stem cell factor (SCF[3]; c-Kit ligand) (2). Stimulation of mast cells by cross-linkage of Fc Ig E receptors (FcεRI) induces the release of granule-associated mediators, such as histamine and tryptase (3, 4), as well as de novo generation of arachidonic acid metabolites and many cytokines, including IL-6 (2, 5, 6). In addition to their well-known role in allergic reactions, mast cells initiate or exacerbate inflammatory processes by secretion of numerous vasoactive and proinflammatory mediators (5). In inflammatory lesions, however, mast cells often do not display signs of explosive degranulation. Growing evidence also indicates that in vivo secretion of the granule-associated mediator tryptase and IL-6 may not always occur in parallel (7). Mouse bone marrow-derived mast cells stimulated with SCF had been shown to secrete IL-6 without any preformed mediators (8).

IL-6 is a multifunctional cytokine that is rapidly elevated in response to inflammatory triggers (9). IL-6 is also elevated and considered important for the development of autoimmune disease and chronic inflammation (9), as well as coronary artery disease (10, 11), where local inflammation (12) and mast cells (13, 14) have been reported to play a role. The proinflammatory cytokine IL-1 is also elevated at inflammatory sites where it regulates gene expression of other inflammatory mediators (15). It appeared to us that IL-1 might have some selective stimulatory effect on mast cells, leading to the production of proinflammatory cytokines. We therefore examined the ability of IL-1 to stimulate the secretion of IL-6 and the release of the granule-associated mast cell protease, tryptase, in human umbilical cord blood-derived mast cells (hCBMCs) and a human leukemic mast cell line (HMC-1). We found that IL-6 was secreted in the absence of degranulation through a new pathway involving 40- to 80-nm vesicles.

Materials and Methods

Cytokines

Human recombinant IL-1α, IL-4, IL-6, and TNF-α were purchased from Chemicon (Temecula, CA). Recombinant human SCF was a gift from Amgen (Thousand Oaks, CA).

Mast cell culture

Human cord blood was obtained from normal vaginal deliveries in accordance with established institutional guidelines. Human CBMCs were derived by the culture of CD34+ progenitor cells as previously described (16) with minor modifications. Briefly, mononuclear cells were isolated by layering heparin-treated cord blood onto Lymphocyte Separation Medium (INC Biomedical, Aurora, OH). CD34+ progenitor cells were isolated from mononuclear cells by selection of cells positive for the AC133 Ag (CD133+/CD34+) by magnetic cell sorting (Miltenyi Biotec, Auburn, CA). For the first 4 wk, CD34+ cells were cultured in IMDM (Life Technologies, Grand Island, NY) supplemented with 0.55 μM 2-ME, 100 mg/liter insulin-transferin-selenium supplement (Life Technologies), 0.1% BSA (Sigma-Aldrich, St. Louis, MO), penicillin/streptomycin, 100 ng/ml of SCF (Amgen), and 50 ng/ml IL-6 (Chemicon) at 37ºC in 5% CO₂-balanced air. After 4 wk of culture, BSA and insulin-transferin-selenium in the culture medium were substituted with 10% FBS (Life Technologies). By 8 wk, 95% of the cells in the culture were identified as mast cells by immunostaining for tryptase. The human leukemic mast cell line (HMC-1) was obtained from Dr. J. H. Butterfield (Mayo Clinic, St. Paul, MN) and was cultured in IMDM supplemented with 10% FBS (HyClone, Logan, UT), penicillin/streptomycin, and 2 μM α-thioglycerol (17).

Mast cell stimulation

For stimulation of IL-6 production, 8- to 14-wk-old hCBMCs were washed three times in PBS and resuspended in fresh medium containing SCF (100 ng/ml) alone, which was included in the stimulation medium in all experiments for optimal mast cell viability. In anti-IgE stimulation experiments, hCBMCs were resuspended (10^6 cells/ml) and passively sensitized by incubation with human myeloma IgE (2 μg/ml/10^6 cells; Chemicon) for 48–72 h in IL-6-free medium at 37ºC. Cells were washed, resuspended in medium containing SFC alone, and distributed to 96-well microtiter assay plates (10^5 cells/200 μl) in duplicate or triplicate for stimulation with IL-1 (50 ng/ml; Chemicon) or anti-IgE (5–10 μg/ml; DAKO, Carpinteria, CA) at 37ºC in 5% CO_2 for 6 h. HMC-1 cells were also distributed to 96-well microtiter assay plates (2×10^5 cells/200 μl) in duplicate or triplicate and stimulated in complete culture medium unless indicated otherwise. For the dose-response of IL-6 and tryptase secretion, hCBMCs and HMC-1 cells were stimulated for 6 h with the indicated concentrations of IL-1. For the time course of IL-6 secretion, hCBMCs were stimulated with 50 ng/ml of IL-1, and HMC-1 were stimulated with 10 ng/ml of IL-1; optimal concentrations were determined from the dose-response experiments. For some experiments hCBMCs were primed with IL-4 (10 ng/ml; Chemicon) for the last 3 wk of culture before stimulation (18). Cells were washed in PBS three times and stimulated with IL-1 (50 ng/ml) in fresh medium containing SCF alone. For determination of whether secreted IL-6 was de novo synthesized, cells were pretreated with the inhibitor of transcription actinomycin D (10 μg/ml; Sigma-Aldrich) or the inhibitor of protein synthesis cycloheximide (15 μg/ml; Sigma-Aldrich) for 30 min at 37ºC before stimulation with IL-1.

IL-6 was determined in cell-free supernatants with commercial ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's directions (sensitivity of the assay, 3 pg/ml). For tryptase measurements, hCBMCs were stimulated for either 6 h in culture medium or 30 min in Tyrode's buffer (133 mM NaCl, 4 mM KCl, 0.64 mM KH_2PO_4, 10 mM HEPES, 1 g/liter glucose, 1 mM $CaCl_2$, 0.6 mM $MgCl_2$, and 0.03% human serum albumin, pH 7.2). Tryptase was measured in cell pellets and supernatants by fluoroenzyme-immunoassay (Amersham Pharmacia Biotech, Uppsala, Sweden).

Data and statistics

IL-6 secretion data are expressed as picograms per 5×10^5 cells for hCBMCs and picograms per 10^6 cells for HMC-1 cells; fewer hCBMCs were used because of their short supply. In IL-4-priming experiments, spontaneous secretion was subtracted from the corresponding stimulated secretion to obtain net IL-6 secretion. Tryptase release data are expressed as nanograms of secreted tryptase per 5×10^5 cells or as the percent release of total tryptase = [supernatant/(supernatant + pellet)] $\times$ 100%. Data represent the mean $\pm$ SEM from three or more experiments, each in duplicate or triplicate and for hCBMCs each experiment done with a culture from different donor. Comparisons between two groups were made with paired t test, and $p < 0.05$ was considered statistically significant.

Calcium measurements

Mast cells (2×10^6 cells/ml) were resuspended in Tyrode's buffer (133 mM NaCl, 4 mM KCl, 0.64 mM KH_2PO_4, 10 mM HEPES, 1 g/liter glucose, 1 mM $CaCl_2$, 0.6 mM $MgCl_2$, and 0.03% human serum albumin, pH 7.2) containing 2.5 mM probenecid and 2 μM cell-permeant fluorescent calcium indicator Calcium Green-1-AM (Molecular Probes, Eugene, OR) for 30 min at room temperature (19). Cells were then washed and resuspended in fresh Tyrode's buffer (10^6 cells/ml). After washes, 2×10^6 cells were placed in a cuvette with constant stirring at 37°C, maintained by a closed circuit, temperature-regulated water pump. Samples were excited at 506 nm, and fluorescence was recorded at 530 nm using a PerkinsElmer fluorescence spectrophotometer (LS-5B; Shelton Chuck, CT). Cells were stimulated with IL-1, anti-IgE, C5a (Sigma-Aldrich) or Br-A23187 (Sigma-Aldrich) as indicated. Data are presented in arbitrary fluorescence units.

Ultrastructural localization of IL-6 and tryptase

Human CBMCs or HMC-1 cells were cultured in the absence or the presence of IL-1 for 18 h (10^6 cells/ml) followed by fixation with 5% acrolein and embedding in LR white (Polysciences, Warrington, PA). Alternatively, cells were fixed with 3% gluteraldehyde/2% paraformaldehyde solution, followed by embedding in epoxy resin without apparent loss of IL-6 antigenicity. Microthin sections (0.1 μm) were cut, mounted on grids, and processed for immunocytochemistry. For IL-6 localization, grids were incubated with 20 μg/ml polyclonal rabbit anti-human IL-6 Ab (US Biologicals, Swampscatt, MA) overnight, followed by goat anti-rabbit IgG conjugated to 10-nm gold particles (1/30; Polysciences). For tryptase

localization, sections were incubated with monoclonal mouse anti-human tryptase Ab (Chemicon) at 40 μg/ml, followed by goat anti-mouse IgG conjugated to 10-nm gold particles (1/30; Polysciences). Sections were then stained with uranyl acetate and Sato's lead. To enhance the visualization of membranes, some samples were postfixed with 3% potassium permanganate (catalog no. P279–500; Fisher Scientific, Suwanee, CA). Sections were then examined using a CM10 transmission electron microscope (Philips, Mahway, NJ).

Results

IL-1 stimulates production of IL-6 without degranulation in hCBMCs

Unstimulated hCBMCs secreted 34 $\pm$ 5.9 ng/5×10^5 of tryptase ($\sim$2–4% of total tryptase), while stimulation of hCBMCs with 10 μg/ml anti-IgE for 6 h resulted in the secretion of 721.1 $\pm$ 90.3 ng/5×10^5 mast cells of tryptase ($n = 6$; $p < 0.05$, by paired t test) or $\sim$55% of the total tryptase (Fig. 1A). Tryptase secretion was similar between control (49.3 $\pm$ 11.6 ng/5×10^5 mast cells) and IL-1-treated (64.7 $\pm$ 18.6 ng/5×10^5 MC) cells. Both, anti-IgE and IL-1 significantly increased IL-6 secretion during a 6-h stimulation (Fig. 1B). IL-6 secretion increased from 5.5 $\pm$ 3.6 pg/5×10^5 cells without IL-1 to 90.5 $\pm$ 20.6 pg/5×10^5 cells in response to IL-1 (50 ng/ml; $n = 6$; $p < 0.05$, by paired t test). Similarly, IL-6 secretion increased from 24.0 $\pm$ 10.2 pg/5×10^5 cells without anti-IgE to 88.2 $\pm$ 18.6 pg/5×10^5 cells in response to anti-IgE (10 μg/ml; $n = 6$; $p < 0.05$, by paired t test). These data demonstrate selective IL-6 secretion in the absence of concomitant tryptase secretion in normal human mast cells.

IL-6 secretion stimulated by IL-1 in hCBMCs and HMC-1 cells

To further characterize IL-1-stimulated IL-6 secretion, dose-response and time-course studies were performed. Stimulation of hCBMCs with IL-1 for 6 h resulted in dose-dependent secretion of IL-6, but not of tryptase (Fig. 2A). Secretion of tryptase (3.5 $\pm$ 0.5%; Fig. 2A) and histamine (data not shown) remained at spontaneous levels at all concentrations of IL-1. Dose-response curves for IL-6 secretion established that optimal responses were obtained with 50 ng/ml of IL-1 for hCBMCs (127.4 $\pm$ 14.8 pg/5×10^5 cells) (Fig. 2A) and with 10 ng/ml for HMC-1 cells (131.0 $\pm$ 5.6 pg/10^6 cells; C).

Next, human mast cells were stimulated with 50 ng/ml (hCBMCs) or 10 ng/ml (HMC-1) of IL-1 for 3, 6, 18, 24, 48, and 72 h. In hCBMCs, the first detectable IL-6 increases were noted 3 h after stimulation, which peaked at 18 h (239.5 $\pm$ 54.4 pg/5×10^5 cells) and remained elevated for the next 72 h (Fig. 2B). In contrast, HMC-1 at 18 h secreted 1762.4 $\pm$ 218.0 pg/10^6 cells IL-6 ($n = 3$; $p < 0.05$), with further increases up to 48 h (Fig. 2D).

We also investigated whether two cytokines together, such as IL-1 and TNF-α, could have a more pronounced effect on IL-6
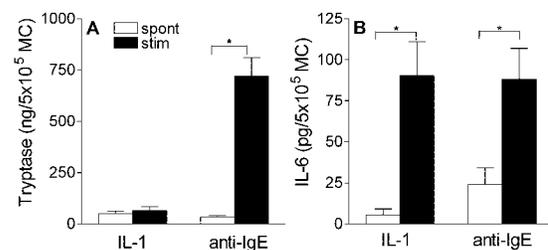

FIGURE 1. IL-1 stimulates IL-6 secretion without degranulation in hCBMCs. Tryptase (*A*) and IL-6 (*B*) secretion by hCBMCs (8–16 wk) in response to stimulation with anti-IgE (10 μg/ml) or IL-1 (50 ng/ml) is shown. Cells were stimulated for 30 min for tryptase measurements and for 6 h for IL-6 measurements. □, Spontaneous secretion; ■, stimulated secretion. Data are the mean $\pm$ SEM ($n = 6$). *, $p < 0.05$, by paired t test.
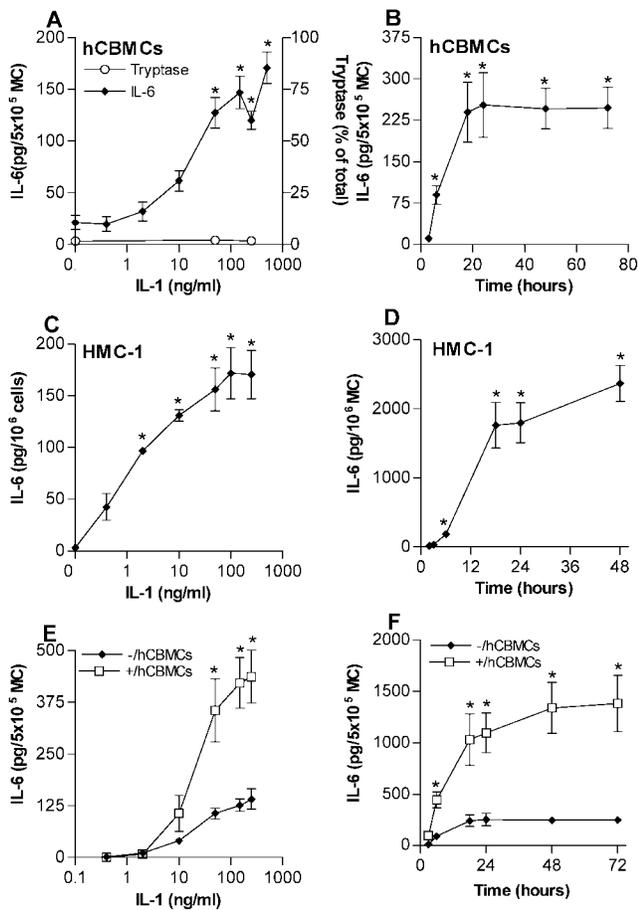
FIGURE 2. Time course and dose response of IL-1-induced secretion of IL-6 from hCBMCs and HMC-1. Tryptase (○) and IL-6 (♦) secretion by hCBMCs (*A*) and HMC-1 (*C*) stimulated with IL-1 for 6 h. The time courses of IL-6 secretion by hCBMCs (*B*) in response to 50 ng/ml of IL-1 and by HMC-1 (*D*) in response to 10 ng/ml of IL-1 are shown. *E*, Dose-response at 6 h; *F*, time course (stimulated with 50 ng/ml IL-1) of IL-6 release from unprimed hCBMCs (−/hCBMCs; ♦) or IL-4-primed hCB-MCs (+/hCBMCs; □). The amount of IL-6 secreted in the absence of IL-1 was subtracted (*E* and *F*) from that of IL-6 secreted in the presence of IL-1 to give net IL-6 secretion. Data are the mean ± SEM ($n = 3$–4). ∗, $p <$ 0.05, by paired t test.

secretion. During an 18-h stimulation of HMC-1 cells, IL-1 (10 ng/ml) alone induced 465.7 ± 38.4 pg/10^6 cells, TNF-α (50 ng/ml) alone induced 99.4 ± 19.2 pg/10^6 cells, and IL-1 and TNF-α together stimulated a synergistic increase of 2329.0 ± 92.6 pg/10^6 cells (mean ± SEM; $n = 3$).

Both hCBMCs and HMC-1 cells are considered to be mucosal-type mast cells. The Th2 cytokine IL-4 has been shown to enhance the maturation of normal human and rodent mast cells associated with increased electron density of the secretory granules (18) and potentiated mediator secretion in response to IgE-dependent stimulation (20–22). We investigated the effect of IL-4 priming on IL-1-stimulated mediator secretion. Eight-week-old hCBMCs were cultured in the absence (−/hCBMCs) or the presence (+/hCBMCs) of IL-4 (10 ng/ml) for 3 wk, washed free of cytokines, and resuspended in stimulation medium free of all cytokines except SCF. Six-hour stimulation of +/hCBMCs with IL-1 (50 ng/ml) resulted in the secretion of a significantly greater amount of IL-6 than that secreted by −/hCBMCs (Fig. 2E; $n = 3$; $p < 0.05$, by paired t test). The time course of IL-1-induced IL-6 secretion by +/hCBMCs (Fig. 2F) resembled that by HMC-1 cells (Fig. 2D), as

IL-6 secreted by +/hCBMCs was 3- to 4-fold more than that by −/hCBMCs (Fig. 2F). Tryptase secretion in response to IL-1 (50 ng/ml) was similar in IL-4-primed hCBMCs (4.3 ± 0.6% of total tryptase) and unprimed hCBMCs (4.4 ± 1.8% of total tryptase) and was similar to baseline secretion in the absence of IL-1.

De novo synthesis of IL-1-induced IL-6 secretion

The delayed time course of IL-6 secretion (3 h earliest increase) and the absence of tryptase secretion suggested that IL-1 stimulated de novo synthesis of IL-6. IL-6 production was completely inhibited (results not shown) by pretreatment (30 min) with either the inhibitor of transcription, actinomycin D (10 μg/ml), or the inhibitor of protein synthesis, cycloheximide (15 μg/ml), in both hCBMCs (100% inhibition; $n = 3$) and HMC-1 (100% inhibition; $n = 5$). Taken together these results confirm that IL-1-induced IL-6 was synthesized de novo.

Calcium requirement for IL-1-induced IL-6 secretion

We further investigated whether the secretion of IL-6 stimulated by IL-1 involved elevation of intracellular calcium ions. Mast cells were first loaded with the cell-permeant, calcium-sensitive fluorescent indicator Calcium Green-1-AM, and fluorescence intensity was measured in cell suspensions. Stimulation of hCBMCs by IL-1 (50 ng/ml) had no effect on intracellular calcium ion levels (Fig. 3A). In contrast, stimulation of IgE-sensitized hCBMCs with anti-IgE (10 μg/ml) resulted in a sustained increase in intracellular calcium ion levels (Fig. 3A). Similarly in HMC-1 cells, stimulation with IL-1 (10 ng/ml) had no effect on intracellular calcium ion levels (Fig. 3B), while stimulation with the complement fragment 5a (C5a, 500 ng/ml), used as a positive control, resulted in transient elevation of intracellular calcium ion levels (Fig. 3B). Subsequent application of a nonfluorescent analog of the cation ionophore, Br-A23187 (0.5 μg/ml), resulted in a further increase in intracellular calcium ion levels (Fig. 3B).

To obtain further confirmation of the lack of a calcium requirement in IL-1-induced IL-6 secretion, hCBMCs or HMC-1 cells were stimulated in a medium containing nominal concentrations of calcium that were obtained by reconstitution of the culture medium in double-distilled water without the addition of calcium. The secretion of IL-6 stimulated by IL-1 in such nominal calcium conditions proceeded unaffected (results not shown).
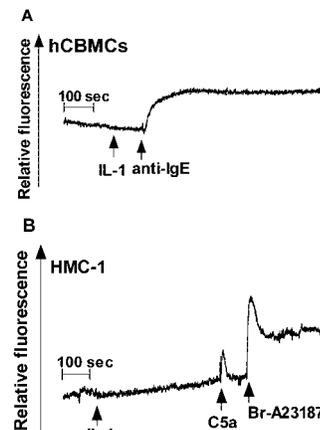

FIGURE 3. Effect of IL-1 on intracellular calcium ion levels. Human CBMCs were stimulated by IL-1 (50 ng/ml), followed by anti-IgE (10 μg/ml; *A*). HMC-1 cells were stimulated with 10 ng/ml IL-1 and 500 ng/ml C5a, followed by 0.5 μg/ml Br-A23187 (nonfluorescent analog of A23187; *B*). Results are presented as arbitrary fluorescence units (au) and are representative of three or more similar experiments.

Ultrastructural localization of IL-6 and tryptase

The ability of IL-1 to induce the secretion of IL-6 without tryptase in the absence of intracellular elevations of calcium ions suggested the involvement of a secretory process other than exocytosis of secretory granules. To visualize the secretory pathway through which IL-6 is secreted, we next investigated the ultrastructural localization of IL-6 and compared it to that of tryptase. Ultrastructural Immunogold labeling showed that the prestored marker tryptase was localized in the secretory granules of hCBMCs (Fig. 4*A*, arrowhead). In contrast, IL-6 was found predominantly in vesicle-like clusters of 5–10 gold particles (Fig. 4*C*); the size of IL-6-containing vesicles (40–80 nm) resembled that of Golgi-derived vesicles. No labeling was observed in negative controls in which the primary Ab for tryptase (Fig. 4*B*) or IL-6 (Fig. 4*D*) was omitted or substituted by nonimmune IgG. These vesicle-like structures were observed in both hCBMCs (Fig. 4*C*, solid arrows) and HMC-1 cells (Fig. 5*A*, solid arrows). It appeared that the number of vesicle-like structures containing IL-6-labeled gold particles was greater in cells stimulated with IL-1 (Fig. 5*A*) than that in unstimulated cells (Fig. 5*B*). Preliminary semiquantitative evaluation of both the vesicle-like structures and the total number of gold-associated particles, however, did not yield statistically significant difference (results not shown). Many of the vesicle-like structures in stimulated cells were located near the plasma membrane or in the immediate extracellular space (Fig. 5*A*, open arrows). Higher magnification identified vesicle-like structures adjacent to the plasma membrane (Fig. 5*C*), budding from the cell surface (Fig. 5*D*), or immediately outside the cell (Fig. 5*E*).

The use of acrolein as a fixative to preserve the antigenicity of IL-6 did not allow adequate preservation of cellular membranes. To better visualize perivesicular membranes, some sections were treated with potassium permanganate. Higher magnifications of select images indicated the presence of a limiting membrane

around a vesicle-like structure containing Immunogold-labeled IL-6 particles (Fig. 5*F*, solid arrowhead).

Discussion

Here we describe for the first time a novel action of the proinflammatory cytokine IL-1 to induce differential release of IL-6 from human mast cells without concomitant release of the preformed mediator tryptase. Both the time course of 3-h delay until the onset of IL-6 secretion and the absence of degranulation were suggestive of vesicular trafficking of newly synthesized, rather than secreted preformed, IL-6. This premise was confirmed by data showing that IL-1-stimulated IL-6 production was completely inhibited by either transcription or protein synthesis inhibitors. These findings also eliminated the possibility that secreted IL-6 was endocytosed by mast cells from the surrounding medium. IL-6 could not be prestored in the granules because assaying the contents of unstimulated cells after sonication gave undetectable levels of IL-6. Similar to our observation that IL-1 did not induce degranulation of human mast cells, IL-1 was previously reported to have no effect on histamine secretion from purified rat peritoneal mast cells (23, 24). Partially purified human adenoidal mast cells were reported to secrete histamine in response to IL-1 (25), but this was most likely an indirect effect through IL-1 activation of contaminating cells. The effects of IL-1 on mast cell cytokine production have not been studied, apart from a few studies showing that IL-1 enhances cytokine production by rodent mast cells previously activated by ionomycin or FcεRI cross-linkage (26) as well as by costimulation with SCF and IL-10 (27). In our study we further showed that IL-6 production stimulated by IL-1 could be potentiated by either costimulation with TNF-α or priming with IL-4 to induce maturation/differentiation of mast cells. Synergistic IL-6 production under these conditions again was in the absence of
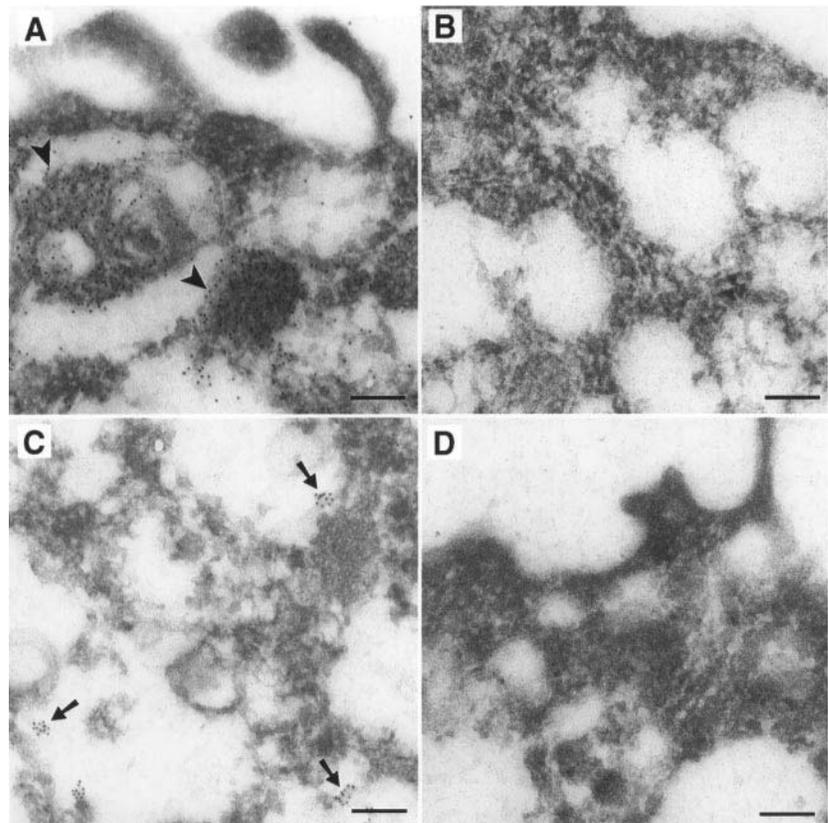
FIGURE 4. Ultrastructural Immunogold localization of IL-6 and tryptase in hCBMCs. Tryptase (arrowhead, *A*) was localized in secretory granules and IL-6 in vesicular clusters (solid arrows, *C*) in hCBMCs. Negative controls, in which the primary Ab for tryptase (*B*) or IL-6 (*D*) was omitted, had no labeling. Scale bars: *A* and *B*, 260 nm; *C* and *D*, 170 nm.
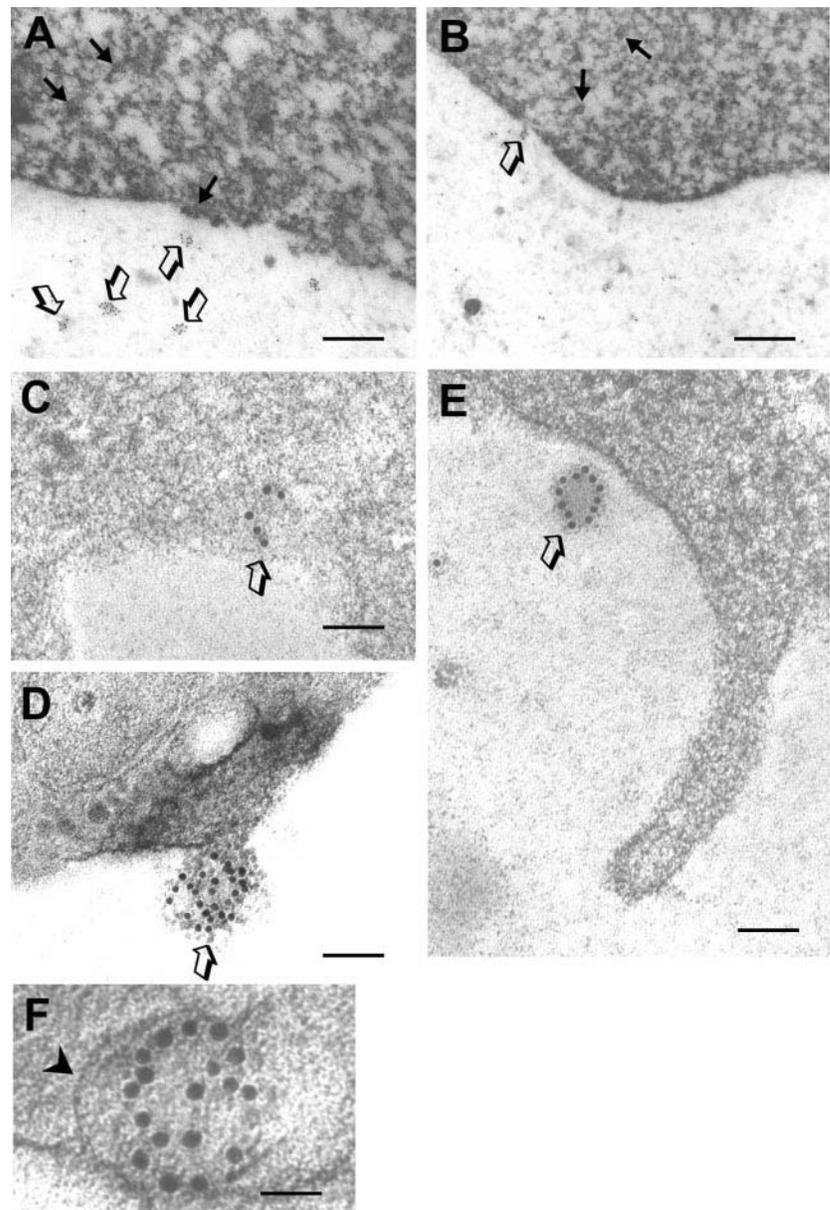
FIGURE 5. Ultrastructural Immunogold localization of IL-6 in HMC-1 cells. IL-6 localization in either acrolein-fixed (*A* and *B*) or aldehyde-fixed (*C–F*) cells with (*D–F*) or without (*A–C*) potassium permanganate treatment. Note the IL-6-containing vesicles inside (solid arrow) and outside (open arrow) an HMC-1 cell stimulated with IL-1 (*A*) or an unstimulated control cell (*B*). *C–E*, A series of higher magnification electron micrographs showing IL-6-containing vesicles (open arrows) in different stages of secretion close to the cell surface: *C*, a vesicle right at the cell surface; *D*, a vesicle budding off from the cell surface; *E*, a vesicle right outside the cell surface with IL-6-associated gold particles evenly distributed in a circular fashion, outlining the shape of the vesicle; *F*, higher magnification of one vesicle where enclosing membrane is visible (arrowhead). Bars: *A* and *B*, 190 nm; *C–E*, 140 nm; *F*, 70 nm.

concomitant degranulation. The ability of rodent mast cells to secrete IL-6 without preformed mediators was reported previously in response to SCF (8) and LPS (28); these agonists, however, were ineffective in stimulating IL-6 production in human mast cells (our unpublished observations) (29).

In further support for a distinct secretory pathway, IL-6 was shown to be excluded from secretory granules and to compartmentalize in vesicle-like structures of 40–80 nm, resembling Golgi-derived secretory vesicles (30). Some stimulated mast cells appeared to contain more IL-6-labeled vesicles. Many vesicles were present at the cell surface and in the immediate extracellular space; the fact that preliminary semiquantitative analysis did not show a statistically significant difference may indicate that there is a much higher rate of vesicular shuttling in IL-1-activated cells than in control cells. The persistence of the electron-dense core of the IL-6-containing clusters outside the cell may act to prevent proteolytic degradation, possibly resulting in longer/greater biological activity. The need to use acrolein to preserve antigenicity made it difficult to identify perivesicular membranes; however, the presence of such membranes could be occasionally discerned at high

magnification, especially after treatment with potassium permanganate. Difficulty in identifying perivesicular membranes in cells preserved for Immunogold labeling is not unique, as it was reported even for neuropeptide-containing vesicles in neurons (31) (T. Hökfelt, unpublished observation). Involvement of the Golgi-vesicular pathway is also supported by a report that secretion of IL-6 induced by the cation ionophore A23187 is sensitive to the fungal metabolite brefeldin A (32), which is known to disrupt vesicle budding from Golgi.

Previous studies have demonstrated that, similar to mast cell degranulation (33), secretion of IL-6 (28) in response to FcϵRI cross-linkage is calcium dependent. In contrast to anti-IgE stimulation of sustained increases in intracellular calcium, IL-1 did not have any effect on intracellular calcium levels in either hCBMCs or HMC-1 cells, suggesting that IL-1-induced IL-6 secretion does not involve the influx of extracellular calcium. This finding was confirmed with subsequent nominal calcium experiments in which IL-6 secretion was unaffected. Alternatively, IL-1 might stimulate calcium influx that is too little or localized too close to the cell surface to be detected with whole cell population measurements

(34). Unlike Fc∈RI-mediated secretion, synthesis of TNF-α in a rat mast cell line was not inhibited by EGTA added 30 min after addition of DNP-BSA (35). In fact, IL-1R activation in most cell types, except fibroblasts (36, 37), is not coupled to calcium influx (38–41); in rare cases where a calcium response to IL-1 may be present in chondrocytes and fibroblasts, it appeared to be dependent on focal cell adhesions (42, 43).

The very first report of differential secretion involved the release of serotonin without histamine in the absence of degranulation from rat peritoneal mast cells (44). However, this differential release of serotonin involved vesicles containing high affinity serotonin binding proteins (45) that appeared to bud off from secretory granules (46). Similarly, mast cells were recently reported to transport fibroblast growth factor in vesicles, but these vesicles also derived from secretory granules (47).

Differential secretion of IL-6 may have gone unnoticed in many inflammatory conditions in which mast cells appear to be involved (3). For instance, it was only recently shown that IL-6 was elevated without concomitant tryptase in suction skin blister fluid (48) and serum (7) of systemic mastocytosis patients, especially those with bone involvement (7). Serum IL-6 levels are increased in patients with rheumatoid arthritis (49, 50), especially those with juvenile rheumatoid arthritis (51, 52). Moreover, IL-6 is required for the development of Ag- and collagen-induced arthritis (53–55). IL-6 is also increased in the serum (10) and coronary sinus (11) of patients with coronary artery disease (56) where numerous mast cells are found around atherosclerotic plaques (13, 57). We recently also showed that cardiac mast cell activation (58) and serum IL-6 increase induced by stress were higher in mice that develop atherosclerosis and could be inhibited by the mast cell stabilizer romolyn (59). Development of inhibitors of IL-1-induced secretion of IL-6 from mast cells could provide novel therapeutic options for the treatment of inflammatory conditions.

Acknowledgments

References

1. Rodewald, H.-R., M. Dessing, A. M. Dvorak, and S. J. Galli. 1996. Identification of a committed precursor for the mast cell lineage. *Science 271:818.*
2. Galli, S. J. 1993. New concepts about the mast cell. *N. Engl. J. Med. 328:257.*
3. Theoharides, T. C. 1996. The mast cell: a neuroimmunoendocrine master player. *Int. J. Tissue React. 18:1.*
4. Serafin, W. E., and K. F. Austen. 1987. Mediators of immediate hypersensitivity reactions. *N. Engl. J. Med. 317:30.*
5. Metcalfe, D. D., D. Baram, and Y. A. Mekori. 1997. Mast cells. *Physiol. Rev. 77:1033.*
6. Kobayashi, H., T. Ishizuka, and Y. Okayama. 2000. Human mast cells and basophils as sources of cytokines. *Clin. Exp. Allergy. 30:1205.*
7. Theoharides, T. C., W. Boucher, and K. Spear. 2002. Serum IL-6 reflects disease severity and osteoporosis in mastocytosis patients. *Int. Arch. Allergy Immunol. 128:344.*
8. Gagari, E., M. Tsai, C. S. Lantz, L. G. Fox, and S. J. Galli. 1997. Differential release of mast cell interleukin-6 via c-kit. *Blood 89:2654.*
9. Papanicolaou, D., R. L. Wilder, S. C. Manolagas, and G. Chrousos. 1998. The pathophysiologic roles of interleukin-6 in human disease. *Ann. Intern. Med. 128:127.*
10. Miyao, Y., H. Yasue, H. Ogawa, I. Misumi, T. Masuda, T. Sakamoto, and E. Morita. 1993. Elevated plasma interleukin-6 levels in patients with acute myocardial infarction. *Am. Heart J. 126.*
11. Deliargyris, E. N., R. J. Raymond, T. C. Theoharides, W. S. Boucher, D. A. Tate, and G. J. Dehmer. 2000. Sites of interleukin-6 release in patients with acute coronary syndromes and in patients with congestive heart failure. *Am. J. Cardiol. 86:913.*
12. Taubes, G. 2002. Does inflammation cut to the heart of the matter? *Science 296:242.*
13. Constantinides, P. 1995. Infiltrates of activated mast cells at the site of coronary atheromatous erosion or rupture in myocardial infarction. *Circulation 92:1083.*
14. Forman, M. B., J. A. Oates, D. Robertson, R. M. Robertson, L. J. Roberts II, and R. Virmani. 1985. Increased adventitial mast cells in a patient with coronary spasm. *N. Engl. J. Med. 313:1138.*
15. Dinarello, C. A. 1996. Biologic basis for interleukin-1 in disease. *Blood 87:2095.*
16. Kempuraj, D., H. Saito, A. Kaneko, K. Fukagawa, M. Nakayama, H. Toru, M. Tomikawa, H. Tachimoto, M. Ebisawa, A. Akasawa, et al. 1999. Characterization of mast cell-committed progenitors present in human umbilical cord blood. *Blood 93:3338.*
17. Butterfield, J. H., D. Weiler, G. Dewald, and G. J. Gleich. 1988. Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leuk. Res. 12:345.*
18. Toru, H., M. Eguchi, R. Matsumoto, M. Yanagida, J. Yata, and T. Nakahata. 1998. Interleukin-4 promotes the development of tryptase and chymase double-positive human mast cells accompanied by cell maturation. *Blood 91:187.*
19. Takahashi, A., P. Camacho, J. D. Lechleiter, and B. Herman. 1999. Measurement of Intracellular calcium. *Physiol. Rev. 79:1089.*
20. Tachimoto, H., M. Ebisawa, T. Hasegawa, T. Kashiwabara, C. Ra, B. S. Bochner, K. Miura, and H. Saito. 2000. Reciprocal regulation of cultured human mast cell cytokine production by IL-4 and IFN-γ. *J. Allergy Clin. Immunol. 106:141.*
21. Yamaguchi, M., K. Sayama, K. Yano, C. S. Lantz, N. Noben-Trauth, J. J. Costa, and S. J. Galli. 1999. IgE enhances Fc∈ receptor I expression and IgE-dependent release of histamine and lipid mediators from human umbilical cord blood-derived mast cells: synergistic effect of IL-4 and IgE on human mast cell Fc∈ receptor I expression and mediator release. *J. Immunol. 162:5455.*
22. Bischoff, S. C., G. Sellge, A. Lorentz, W. Sebald, R. Raab, and M. P. Manns. 1999. IL-4 enhances proliferation and mediator release in mature human mast cells. *Proc. Natl. Acad. Sci. USA 96:8080.*
23. Otsuka, H., K. Abe, and M. Oka. 1992. Effects of human recombinant IL-1 on *d*-tubocurarine-induced histamine release from isolated rat peritoneal mast cells. *Agents Actions 36:178.*
24. Lukacs, N. W., S. L. Kunkel, R. M. Strieter, H. L. Evanoff, R. G. Kunkel, M. L. Key, and D. D. Taub. 1996. The role of stem cell factor (c-kit ligand) and inflammatory cytokines in pulmonary mast cell activation. *Blood 87:2262.*
25. Subramanian, N., and M. A. Bray. 1987. Interleukin 1 releases histamine from human basophils and mast cells *in vitro. J. Immunol. 138:271.*
26. Hultner, L., S. Kolsch, M. Stassen, U. Kaspers, J. P. Kremer, R. Mailhammer, J. Moeller, H. Broszeit, and E. Schmitt. 2000. In activated mast cells, IL-1 up-regulates the production of several Th2-related cytokines including IL-9. *J. Immunol. 164:5556.*
27. Lu-Kuo, J. M., K. F. Austen, and H. R. Katz. 1996. Post-transcriptional stabilization by interleukin-1β of interleukin-6 mRNA induced by c-kit ligand and interleukin-10 in mouse one marrow-derived mast cells. *J. Biol. Chem. 271:22169.*
28. Leal-Berumen, I., P. Conlon, and J. S. Marshall. 1994. IL-6 production by rat peritoneal mast cells is not necessarily preceded by histamine release and can be induced by bacterial lipopolysaccharide. *J. Immunol. 152:5468.*
29. McCurdy, J. D., T. J. Olynych, L. H. Maher, and J. S. Marshall. 2003. Cutting edge: distinct Toll-like receptor 2 activators selectively induce different classes of mediator production from human mast cells. *J. Immunol. 170:1625.*
30. Rothman, J. E. 1994. Mechanisms of intracellular protein transport. *Nature 372:55.*
31. Bean, A. J., X. Zhang, and T. Hokfelt. 1994. Peptide secretion: what do we know?. *FASEB J. 8:630.*
32. Zhu, F., K. Gomi, and J. S. Marshall. 1998. Short-term and long-term cytokine release by mouse bone marrow mast cells and the differentiated KU-812 cell line are inhibited by brefeldin A. *J. Immunol. 161:2541.*
33. Douglas, W. W., and M. Kagayama. 1977. Calcium and stimulus-secretion coupling in the mast cell: stimulant and inhibitory effects of calcium-rich media on exocytosis. *J. Physiol. 270:691.*
34. Theoharides, T. C., and W. W. Douglas. 1978. Secretion in mast cells induced by calcium entrapped within phospholipid vesicles. *Science 201:1143.*
35. Buccione, R., S. Bannykh, I. Santone, M. Baldassarre, F. Facchiano, Y. Bozzi, G. Di Tullio, A. Mironov, A. Luini, and M. A. De Matteis. 1996. Regulation of constitutive exocytotic transport by membrane receptors: a biochemical and morphometric study. *J. Biol. Chem. 271:3523.*
36. Corkey, B. E., J. F. Geschwind, J. T. Deeney, D. E. Hale, S. D. Douglas, and L. Kilpatrick. 1991. Ca²⁺ responses to interleukin 1 and tumor necrosis factor in cultured human skin fibroblasts: possible implications for Reye syndrome. *J. Clin. Invest. 87:778.*
37. Bouchelouche, P. N., C. Reimert, and K. Bendtzen. 1988. Effects of natural and recombinant interleukin-1α and -β on cytosolic free calcium in human and murine fibroblasts. *Leukemia 2:691.*
38. Bathon, J. M., J. C. Croghan, D. W. MacGlashan, and D. Proud. 1994. Bradykinin is a potent and relatively selective stimulus for cytosolic calcium elevation in human synovial cells. *J. Immunol. 153:2600.*
39. Abraham, R. T., S. N. Ho, T. J. Barna, and D. J. McKean. 1987. Transmembrane signaling during interleukin 1-dependent T cell activation. Interactions of signal 1-and signal 2-type mediators with phosphoinositide-dependent signal transduction mechanism. *J. Biol. Chem. 262:2719.*
40. Georgilis, K., C. Schaefer, C. A. Dinarello, and M. S. Klempner. 1987. Human recombinant interleukin 1β has no effect on intracellular calcium or on functional responses of human neutrophils. *J. Immunol. 138:3403.*
41. Stanton, T. H., M. Maynard, and K. Bomsztyk. 1986. Effect of interleukin-1 on intracellular concentration of sodium, calcium, and potassium in 70Z/3 cells. *J. Biol. Chem. 261:5699.*

42. Luo, L., T. Cruz, and C. McCulloch. 1997. Interleukin 1-induced calcium signalling in chondrocytes requires focal adhesions. *Biochem. J. 324:653.*

43. Arora, P. D., J. Ma, W. Min, T. Cruz, and C. A. McCulloch. 1995. Interleukin-1-induced calcium flux in human fibroblasts is mediated through focal adhesions. *J. Biol. Chem. 270:6042.*

44. Theoharides, T. C., P. K. Bondy, N. D. Tsakalos, and P. W. Askenase. 1982. Differential release of serotonin and histamine from mast cells. *Nature 297:229.*

45. Tamir, H., T. C. Theoharides, M. D. Gershon, and P. W. Askenase. 1982. Serotonin storage pools in basophil leukemia and mast cells: characterization of two types of serotonin binding protein and radioautographic analysis of the intracellular distribution of [^{3}H]serotonin. *J. Cell Biol. 93:638.*

46. Kops, S. K., T. C. Theoharides, C. T. Cronin, M. G. Kashgarian, and P. W. Askenase. 1990. Ultrastructural characteristics of rat peritoneal mast cells undergoing differential release of serotonin without histamine and without degranulation. *Cell Tissue Res. 262:415.*

47. Dvorak, A. M., E. S. Morgan, and P. F. Weller. 2001. Ultrastructural immunolocalization of basic fibroblast growth factor to lipid bodies and secretory granules in human mast cells. *Histochem. J. 33:397.*

48. Brockow, K., C. Akin, M. Huber, L. M. Scott, L. B. Schwartz, and D. D. Metcalfe. 2002. Levels of mast-cell growth factors in plasma and in suction skin blister fluid in adults with mastocytosis: correlation with dermal mast-cell numbers and mast-cell tryptase. *J. Allergy Clin. Immunol. 109:82.*

49. Hirano, D., M. Nagashima, R. Ogawa, and S. Yoshino. 2001. Serum levels of interleukin 6 and stress related substances indicate mental stress condition in patients with rheumatoid arthritis. *J. Rheumatol. 28:490.*

50. Wellby, M. L., J. A. Kennedy, K. Pile, B. S. True, and P. Barreau. 2001. Serum interleukin-6 and thyroid hormones in rheumatoid arthritis. *Metabolism 50:463.*

51. Pignatti, P., M. Vivarelli, C. Meazza, M. G. Rizzolo, A. Martini, and F. De Benedetti. 2001. Abnormal regulation of interleukin 6 in systemic juvenile idiopathic arthritis. *J. Rheumatol. 28:1670.*

52. Shahin, H. A., A. A. Shahin, O. G. Shaker, N. Kamal, H. A. Hafez, and W. Gaber. 2002. Circulating interleukin-6, soluble interleukin-2 receptors, tumor necrosis factor α, and interleukin-10 levels in juvenile chronic arthritis: correlations with soft tissue vascularity assessed by power Doppler sonography. *Rheumatol. Int. 22:84.*

53. Boe, A., M. Baiocchi, M. Carbonatto, R. Papoian, and O. Serlupi-Crescenzi. 1999. Interleukin 6 knock-out mice are resistant to antigen-induced experimental arthritis. *Cytokine 11:1057.*

54. Alonzi, T., E. Fattori, D. Lazzaro, P. Costa, L. Probert, G. Kollias, F. De Benedetti, V. Poli, and G. Ciliberto. 1998. Interleukin 6 is required for the development of collagen-induced arthritis. *J. Exp. Med. 187:461.*

55. Ohshima, S., Y. Saeki, T. Mima, M. Sasai, K. Nishioka, S. Nomura, M. Kopf, Y. Katada, T. Tanaka, M. Suemura, et al. 1998. Interleukin 6 plays a key role in the development of antigen-induced arthritis. *Proc. Natl. Acad. Sci. USA 95:8222.*

56. Yudkin, J. S., M. Kumari, S. E. Humphries, and V. Mohamed-Ali. 2000. Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? *Atherosclerosis 148:209.*

57. Jeziorska, M., C. McCollum, and D. E. Woolley. 1997. Mast cell distribution, activation and phenotype in atherosclerotic lesions of human carotid arteries. *J. Pathol. 182:115.*

58. Theoharides, T. C. 2002. Mast cells and stress: a psychoneuroimmunological perspective. *J. Clin. Psychopharmacol. 22:103.*

59. Huang, M., X. Pang, K. Karalis, and T. C. Theoharides. 2003. Stress-induced interleukin-6 release in mice is mast cell-dependent and more pronounced in apolipoprotein E knockout mice. *Cardiovasc. Res. 59:241.*