Release of Both Preformed and Newly Synthesized Tumor Necrosis Factor \(\alpha\) (TNF-\(\alpha\))/Cachectin by Mouse Mast Cells Stimulated via the Fc\(_\varepsilon\)RI.

A Mechanism for the Sustained Action of Mast Cell-derived TNF-\(\alpha\) during IgE-dependent Biological Responses

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Summary

Mast cell-associated mediators are generally classified into two groups: the preformed mediators, which are stored in the cells’ cytoplasmic granules and are released upon exocytosis, and the newly synthesized mediators, which are not stored but are produced and secreted only after appropriate stimulation of the cell. We now report that tumor necrosis factor \(\alpha\) (TNF-\(\alpha\))/cachectin represents a new type of mast cell-associated mediator, in that IgE-dependent mast cell activation results in the rapid release of preformed stores of the cytokine followed by the synthesis and sustained release of large quantities of newly formed TNF-\(\alpha\). We also demonstrate that challenge with specific antigen induces higher levels of TNF-\(\alpha\) mRNA at skin sites sensitized with IgE in normal mice or mast cell-reconstituted genetically mast cell-deficient WBB6F\(_1\)-W/W\(^{\text{v}}\) mice than at identically treated sites in WBB6F\(_1\)-W/W\(^{\text{v}}\) mice that are devoid of mast cells. These findings identify mast cells as a biologically significant source of TNF-\(\alpha\)/cachectin during IgE-dependent responses and define a mechanism whereby stimulation of mast cells via the Fc\(_\varepsilon\)RI can account for both the rapid and sustained release of this cytokine.
Materials and Methods

Cells. The derivation of the IL-3-independent cloned mast cell line CI,MC/C57.1 has been reported (12). Primary cultures of IL-3-dependent, bone marrow-derived cultured mast cells (BMMC) and purified peritoneal mast cells (PMC) were prepared from retired breeder BALB/c mice (6). BMMC (>95% mast cells by staining with neutral red) were used 4-5 wk after the initiation of culture (6). Purified PMC contained >98% mast cells according to neutral red staining (6). The L929 cell line was obtained and maintained as reported (6, 12). For FeRI-dependent activation, mast cells were sensitized with a monoclonal (mc) IgE anti-dinitrophenol (DNP) antibody (13) for 3 h (6). The CI,MG/C57.1 and BMMC were then stimulated with DNP6-4-HSA (50 ng/ml; Sigma Chemical Co., St. Louis, MO) (5, 6), whereas PMC were challenged with a rat mc anti–mouse IgE (150 ng/ml final concentration) (6). In experiments measuring the release of preformed, as opposed to newly synthesized TNF-α, the transcription inhibitor actinomycin D (act D, 10 μg/ml; Sigma Chemical Co.) was added to the cultures 5-10 min before activation of the cells with antigen or anti-IgE.

Measurement of TNF-α Secretion. TNF-α bioactivity in mast cell supernatants was determined using an MTT 18-h cytotoxicity assay with L929 target cells (6). The specificity of the cytotoxic activity was determined by neutralization with a monospecific rabbit anti-TNF-α antisera (6), and recombinant murine TNF-α (Genzyme, Boston, MA) standards were included in each assay. Statistical analysis of the data was performed using a two-tailed Students t test.

Serotonin Release Assay. To evaluate the release of a classical preformed mediator, we monitored the kinetics of release of the cytoplasmic granule–associated mediator serotonin (5-hydroxytryptamine; 5-HT) (14). Briefly, mc IgE anti-DNP and 5-[1,2-3H](N)-5-hydroxytryptamine creatinine sulfate (New England Nuclear, Boston, MA) (sp act, 25.2 Ci/mmol, 1 μCi/ml) were added to the mast cells for 3 h, then the cells were washed and challenged with antigen (CI,MG/C57.1 and BMMC) or mc anti-IgE (PMC) as noted above. The percent specific [3H]5-HT release was calculated (14) using the formula: percent specific release = 100 × [(cpm stimulated release/cpm stimulated cells + supernatant) – (cpm unstimulated release/cpm unstimulated cells + supernatant)].

Isolation of RNA. RNA was obtained from the cultured cells as described (5, 6) and from mouse skin specimens after homogenization in guanidinium isothiocyanate solution (5, 6) using a tissue homogenizer (Polytron, Kinematica GmbH Littau, Switzerland). The homogenates were cleared by centrifugation for 30 min at 10,000 g, and the cellular RNA was isolated (5, 6), and the mRNA was purified by oligo d(T) cellulose chromatography (6).

Northern Blotting of RNA. Briefly, the RNA was size fractionated on formaldehyde-agarose gels and transferred to nylon membranes (Zeta-bind; Cuno Laboratories, Meriden, CT). The blots were hybridized with 32P-labeled specific cDNA probes, washed at high stringency, and photographed (6). In some experiments, the signals on the autoradiographs were quantitated by laser densitometry, and the results corrected for variation in RNA loading, as determined by reference to control (actin) cDNA probes.

Molecular Probes for Detection of Cytokine mRNA. The 1-kb TNF-α cDNA probe (15), an EcoRI fragment from a plasmid generously provided by Dr. Michael Palladino (Genentech, Inc., South San Francisco, CA), was body labeled with [35S]dATP using the random hexamer priming method. A synthetic oligonucleotide probe (sequence: 5′-GTCGCCCTTTACTCAAGCTTTTGAC-3′) specific for exon 4 of murine TNF-α (16) was generously provided by Dr. Glen Andrews (Pfizer Pharmaceuticals, Groton, CT) and was 3′ labeled with [32P]ATP using the terminal deoxynucleotidyl transferase reaction. Both probes were used as noted (6).

Determination of TNF-α mRNA Half-Life. Briefly, 60-90 min after immunologic activation of the mast cells, act D (10 μg/ml) was added to the cell cultures to block nascent transcription, and at varying intervals thereafter, the residual cellular mRNA was extracted from the cells and analyzed by Northern blotting. The amount of specific mRNA was quantified by laser densitometry (above) and the biologic half-life (t1/2) determined from the graphed results.

Induction of Passive Cutaneous Anaphylaxis (PCA) Responses. Briefly, we injected ~48 ng of mc IgE anti-DNP antibody in 20 μl of H esep-buffered HBSS (Hepes-HBSS) intradermally into the ears. After 24 h, the mice were challenged intravenously with 100 μg of DNP6-4-HSA (Sigma Chemical Co.) in 200 μl of Hepes-HBSS containing 0.5% Evan’s blue dye. The presence of PCA reactivity was confirmed by measuring the ear swelling (Δ ear thickness) and/or by assessing the extravasation of Evan’s blue dye (17). At 45 min after antigen challenge, ear biopsies were processed for RNA extraction and Northern analysis as noted above.

Local Cutaneous Mast Cell Reconstitution of W/W° Mice. The chronic application of PMA to the skin of genetically mast cell–deficient W/W° mice induces the development of large numbers of phenotypically normal dermal mast cells that are competent to orchestrate PCA reactions (17). This effect is localized to the PMA-treated site; mast cells do not develop at contralateral sites treated with vehicle (acetone) alone (17). The mast cells that develop at PMA-treated sites persist for at least 8 wk after the PMA treatments are stopped and the local inflammatory responses have waned (our unpublished results). For the experiments reported here, we induced mast cell development in the ear skin of W/W° mice by chronic application of PMA (5 μg/site in 5 μl of acetone, three times per week for 6 wk); the contralateral ears were treated on the same schedule with acetone alone (17). The animals were then left untreated for an additional 6-8 wk, to permit the local inflammatory reactions to subside.

Results and Discussion

Kinetics of Induction of TNF-α/Cachectin mRNA in Mast Cells Stimulated via the FcRI. We first determined the kinetics of TNF-α mRNA induction in CI,MG/C57.1 mast cells stimulated via the FcRI (Fig. 1). TNF-α mRNA increased to very high levels by 30-60 min after challenge, then gradually waned by 4-6 h after stimulation. We showed that the signals in these plots indeed represented TNF-α mRNA by probing similar blots with an oligonucleotide probe specific for exon 4 of TNF-α (Fig. 1). Primary populations of IL-3-dependent BMMC stimulated with IgE and specific antigen exhibited kinetics of TNF-α mRNA induction that were the same as those observed with CI,MG/C57.1 mast cells (data not shown). The t1/2 of TNF-α mRNA in either CI,MG/C57.1 cells or BMMC was ~35 min.

Kinetics of Release of Preformed or Newly Synthesized TNF-α/Cachectin by Mast Cells. To assess the contribution of preformed as opposed to newly synthesized TNF-α/cachectin...
Figure 1. Northern blot analysis of the kinetics of induction of TNF-α/cachectin mRNA in mast cells stimulated via the FcERI. C1.MC/C57.1 cells were sensitized with mc IgE anti-DNP antibody, washed, and then challenged with DNP30-40-HSA. At the times shown, total cellular RNA was extracted from the cells and processed for Northern blotting. The upper panel is an autoradiograph of a Northern blot probed with a 32P-labeled 1-kb TNF-α cDNA probe and a UV photograph prepared of the ethidium bromide-stained RNA in the gel before blotting. The lower panel is an autoradiograph of a Northern blot similar to that in upper panel, but probed with a 32P-labeled 30-base synthetic oligonucleotide probe specific for exon 4 of TNF-α.

to the cytokine bioactivity released upon IgE-dependent mast cell stimulation, we activated the cells either in the presence (+) or absence (-) of act D (Fig. 2). Identically treated mast cells that first had been permitted to incorporate [3H]5-HT were also tested, to determine whether act D treatment influenced release of the cytoplasmic granule-associated preformed mediator 5-HT (Fig. 2). In C1.MC/C57.1 mast cells tested in the absence of act D (Fig. 2 a), release of [3H]5-HT reached maximal levels within 10 min of antigen challenge, whereas release of TNF-α bioactivity did not achieve maximal levels until 60 min after challenge. In the presence of act D (Fig. 2 b), the C1.MC/C57.1 mast cells released substantially less TNF-α bioactivity, which reached maximal levels by 10 min and actually declined thereafter (Fig. 2 b). Note that data for TNF-α release in Fig. 2, a and b are shown on a log scale; maximal levels of release of preformed TNF-α (Fig. 2 b) represented only 10% of the maximal levels of cytokine released by cells stimulated in the absence of act D (Fig. 2 a). Similar results were obtained with BMCMC and purified PMC (Fig. 2 c).

Taken together, these experiments show that in cloned mast cells, primary cultures of immature IL-3-dependent mast cells, and purified mature PMC, FcERI-dependent activation results in the rapid release of preformed stores of TNF-α/cachectin followed by the sustained release of substantially greater amounts of newly formed cytokine. In contrast to its dramatic effect on TNF-α release, incubation with act D had no discernable effect (p > 0.05 at all time intervals) on IgE-dependent release of [3H]5-HT from any of the mast cell populations (Fig. 2, a–c). The latter results indicated that the effect of act D on TNF-α release did not reflect an impairment of mast cell exocytosis per se, but instead was due to its ability to block the synthesis of TNF-α in response to IgE-dependent stimulation of the cells.

Mast Cells Represent a Major Source of TNF-α mRNA In Vivo. To determine if mast cells represented a significant source of TNF-α during IgE-dependent immunological responses in vivo, we searched for TNF-α mRNA in the skin of normal (WBB6F1/+) mice, genetically mast cell–deficient WBB6F1/Wmice, and mast cell–reconstituted Wmice after sensitization of the cutaneous sites with mc IgE anti-DNP and intravenous challenge of the animals 24 h later with DNP-HSA (Fig. 3). As reported previously (17, 18), significant tissue swelling occurred in association with 30-min PCA reactions in +/+ mice but not at identi-
Figure 3. TNF-α/cachectin mRNA induction after specific immunologic challenge of IgE-sensitized skin in mice is mast cell dependent. (a) Left panel, IgE-dependent dermal inflammatory responses in IgE-sensitized skin of genetically mast cell-deficient WBB6F1-W/W (W/Wv) mice or the congenic normal WBB6F1-+/+ (+/+) mice, as measured by ear-swelling 30 min after intravenous antigen challenge. (Right panel) Relative TNF-α mRNA levels 45 min after antigen challenge in biopsies of cutaneous reaction sites obtained from the W/Wv and +/+ mice shown in the left panel, as determined by laser densitometric scanning of Northern blot autoradiographs. The levels of actin mRNA in the biopsies were used as internal standards to control for RNA loading on the Northern blots (see Materials and Methods). (b) Mast cell reconstitution of one ear of W/Wv mice repairs the defect in IgE-dependent TNF-α mRNA in the mast cell-reconstituted ears, but not in the contralateral, mast cell-deficient ears. (Left panel) Northern blot analysis of the TNF-α and actin mRNA levels within reaction sites in mast cell-deficient (Defic.) ears and the contralateral mast cell-reconstituted (Recon.) ears of W/Wv mice (~5 μg of oligo-dT-selected mRNA/lane). (Right panel) Graphic presentation of relative TNF-α mRNA levels in the mast cell-deficient and mast cell-reconstituted reaction sites, as determined by laser densitometric analysis of the Northern blot in left panel. As in a, the relative TNF-α mRNA content was corrected for variation in mRNA loading, by reference to the actin signals.
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