Mast Cell Participation During the Elicitation of Murine Allergic Contact Hypersensitivity*

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In order to evaluate mast cell participation in allergic contact hypersensitivity (ACH), BALB/c mice were sensitized with 0.1% trinitrochlorobenzene (TNCB). Immediately before challenge and at 1, 1.5, 2, 4, 6, 8, 12, 24, and 48 h after challenge with 1% TNCB, groups of animals had ear thickness measured, had blood collected for histamine determinations, and had both ears removed for histologic evaluation of mast cells.

The increase in ear swelling was triphasic with peak increases at 1.5 h (14.3 ± 1.6 × 10−2 mm; mean ± SEM), 8 h (19.9 ± 1.8 × 10−2 mm), and 24 h (30.2 ± 2.9 × 10−2 mm). A triphasic pattern of increased serum histamine was noted at 1–4 h (117% over control levels), at 12 h (131%), and at 48 h (133%). Examination of the tissue specimens from challenged animals showed modest (1+) degradation of mast cells between 1 and 6 h with extensive (2+) degradation at 12 h. In addition, hypogranulated mast cells were evident between 1 and 6 h, at 24 h, and at 48 h. There were no statistically significant differences in mast cell numbers at any time. Neither platelets nor other formed elements of the blood contributed to the increased blood histamine levels. These data show that mast cells are activated in a triphasic pattern during ACH, and thus suggest both early and late roles for the mast cell and its products in the evolution of ACH. J Invest Dermatol 88: 686–690, 1987

The role of the mast cell and its mediators in allergic contact hypersensitivity (ACH) remains controversial [1–3]. Although prior observations [4–6] have suggested the involvement of this cell in ACH, the necessity of the mast cell has recently been questioned by studies showing no decrease in ACH in mutant mice congenitally deficient in mast cells [2,7,8]. Additionally, reserpine, an agent that depletes intracellular serotonin in mast cells, which was used in many of the early ACH experiments [4], has been found to have additional effects on cell-mediated immunologic reactions [9].

The aim of this study was to assess the participation of mast cells by measuring serum histamine as a direct indicator of mast cell degranulation during ACH and to correlate these levels with clinical ear swelling measurements and histologic examination of the affected tissues for evidence of mast cell degranulation.

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Elicitation of Contact Hypersensitivity Groups of mice were sensitized by applying 100 μl of 0.1% trinitrochlorobenzene (TNCB) (King’s Laboratory Inc., Blythewood, South Carolina) diluted in acetone and olive oil (4:1) to the dorsal neck region on day zero. On day 7, the mice were challenged by applying 20 μl of 1% TNCB diluted in acetone and olive oil (4:1) to the dorsal surface of one ear. Immediately prior to, and at 1, 1.5, 2, 4, 6, 8, 12, 24, and 48 h after challenge, groups of animals were anesthetized with ether; ear-swelling responses were measured using an engineer's micrometer (Mitutoyo Mfg. Co. Ltd., Tokyo, Japan). The ear swelling response was calculated by subtracting the thickness of the ear at time 0 h from the thickness of the ear at the various times examined after challenge. Blood was collected by lacerting the dorsal tail vein, and the ears were removed for histologic examination. In order to note the challenged side of the ear in tissue sections, the dorsal aspect of the ear was painted with blue tissue stain (Dermatologic Lab and Supply Co., Council Bluffs, Iowa). Eight individual mice were studied at each time point. Normal animals, animals sensitized but not challenged, and animals not sensitized but challenged were used as controls. Animals that were sensitized but not challenged failed to develop any clinical reaction at days 5, 6, or 7 post sensitization. In addition, serum histamine levels were no different from normal controls and therefore these mice were not examined at all the time points (data not shown). Eight mice that were neither sensitized nor challenged were used as the normal controls and provided the mean base line serum histamine level. A total of 20 mice that were challenged without prior sensitization were used as irritant controls; these were studied at all the time points with 2–4 animals at each time point.

MATERIALS AND METHODS

Animals Ten- to twelve-week-old BALB/c mice weighing 19–21 g were obtained from Taconic Laboratories (Germantown, New York).

Elicitation of Contact Hypersensitivity Groups of mice were sensitized by applying 100 μl of 0.1% trinitrochlorobenzene (TNCB) (King’s Laboratory Inc., Blythewood, South Carolina) diluted in acetone and olive oil (4:1) to the dorsal neck region on day zero. On day 7, the mice were challenged by applying 20 μl of 1% TNCB diluted in acetone and olive oil (4:1) to the dorsal surface of one ear. Immediately prior to, and at 1, 1.5, 2, 4, 6, 8, 12, 24, and 48 h after challenge, groups of animals were anesthetized with ether; ear-swelling responses were measured using an engineer’s micrometer (Mitutoyo Mfg. Co. Ltd., Tokyo, Japan). The ear swelling response was calculated by subtracting the thickness of the ear at time 0 h from the thickness of the ear at the various times examined after challenge. Blood was collected by lacerting the dorsal tail vein, and the ears were removed for histologic examination. In order to note the challenged side of the ear in tissue sections, the dorsal aspect of the ear was painted with blue tissue stain (Dermatologic Lab and Supply Co., Council Bluffs, Iowa). Eight individual mice were studied at each time point. Normal animals, animals sensitized but not challenged, and animals not sensitized but challenged were used as controls. Animals that were sensitized but not challenged failed to develop any clinical reaction at days 5, 6, or 7 post sensitization. In addition, serum histamine levels were no different from normal controls and therefore these mice were not examined at all the time points (data not shown). Eight mice that were neither sensitized nor challenged were used as the normal controls and provided the mean base line serum histamine level. A total of 20 mice that were challenged without prior sensitization were used as irritant controls; these were studied at all the time points with 2–4 animals at each time point.

Abbreviations:
ACH: allergic contact hypersensitivity
TNCB: trinitrochlorobenzene
**Assay for Histamine**  Normal mice were bled into tubes coated with citrate anticoagulant, pH 5.2. The tubes were spun either at 200 g to obtain platelet-rich plasma or at 8800 g to obtain platelet-poor plasma. Histamine content of the platelet-rich and platelet-poor plasmas after 10 min of boiling was assessed to determine the histamine concentration of mouse platelets. In separate experiments normal mice were bled into tubes coated with citrate anticoagulant, pH 5.2, which were spun at 8800 g. The aspirated buffy coat was washed 3 times with 10 vol of 0.1 M phosphate-buffered saline, pH 7.9; after boiling for 10 min, the histamine content was assessed. Blood was allowed to clot in Eppendorf tubes (VWR Scientific, South Plainfield, New Jersey) and spun at 8800 g for 15 min; the serum was collected and stored at −20°C.

Histamine was measured using a previously described method [10]. Briefly, a 100-μl aliquot of serum, plasma, or cells was added to a mixture containing [3H]-histamine (7.8 Ci/mmol) (New England Nuclear, Boston, Massachusetts), 14C-S-adenosyl methionine (59.8 mCi/mmol) (New England Nuclear), and adequate amounts of histamine-N-methyltransferase prepared from rat kidney; samples were incubated for 90 min at 37°C, and the reaction was then terminated by adding 4% perchloric acid. After extraction in chloroform and NaOH, the samples were quantified in 5 ml of Aquasol (New England Nuclear) in a Packard PLD Tri-carb liquid scintillation counter (Packard Instruments, Downers Grove, Illinois). The sensitivity of this assay is as low as 1 ng/ml.

**Histologic Evaluation** Upon removal, the ears were embedded in OCT compound (Miles Scientific, Naperville, Illinois) and frozen. Four-micron frozen sections were prepared, placed in 95% ethyl alcohol, rinsed in distilled water, and stained with Giemsa reagent (Fisher Scientific, Fairlawn, New Jersey). The slides were dipped in tap water, dehydrated through 95% to 100% ethyl alcohol, cleared in Xylene, and mounted with Permount (Fisher Scientific). The entire cross section of the ear was examined microscopically by 2 independent observers in a blinded fashion. The state of granulation of mast cells was graded on a semi-quantitative scale of 0 to 2+, in which 0 denoted intact mast cells; +/− denoted few granules outside of the cytoplasm of some of the cells, but the majority of the cells remained intact; 1+ denoted scattered granules outside the cells in a patchy distribution, but with more than 50% of the cells intact; and 2+ denoted confluent degranulation observed throughout the ear with the majority of the cells displaying some degree of degranulation. The amount of degranulation observed in the unchallenged ear was subtracted from that of the challenged ear. In addition, cells in which the density of the granules was decreased and in which the nucleus was clearly visible were designated hypogranulated.

The number of mast cells in 6 consecutive lengths of the ear from epidermis to cartilage was counted using a calibrated grid 0.25 μm in length (Carl Zeiss, Inc., New York, New York). Histologic evaluation was conducted on 3 mice at each time point for sensitized and challenged animals and on the irritant controls.

**Statistics** Significant differences between the means and standard error were determined using a Student’s t-test.

**RESULTS**

**Ear Swelling** The ear swelling response was triphasic with peaks at 1.5, 8, and 24 h (Fig 1). The increments were 14.3 ± 1.6 × 10⁻² mm (mean ± SEM), 19.9 ± 1.8 × 10⁻² mm, and 30.2 ± 2.9 × 10⁻² mm, respectively. Mice sensitized but not challenged failed to show any ear swelling. Challenged but unsensitized mice developed an irritant ear swelling response, which was maximal at 12 h (11.3 ± 5.2 × 10⁻² mm).

**Serum Histamine** The level of serum histamine in the normal control mice was 46.4 ± 4.8 ng/ml (mean ± SEM). Increased histamine levels occurred in a triphasic fashion with peaks at 1–4, 12, and 48 h (Fig 2). The increments (Δ) were 54.3 ± 9.4 ng/ml (p < 0.001), 60.8 ± 11.9 ng/ml (p < 0.001), and 61.7 ± 24.8 ng/ml (p < 0.02), respectively. These values represent 117%, 131%, and 133% increases above control levels. Mice sensitized but not challenged failed to develop any statistically significant increase in serum histamine either on days 5, 6, or 7 post sensitization (data not shown). The irritant controls also failed to develop a consistent or significant elevation in serum histamine. The mean histamine for these animals at all time points was 42.8 ± 4.6 ng/ml. Plasma rich in platelets and poor in platelets failed to show any difference in histamine content. The formed elements of the blood contained only small amounts of histamine ranging from 1.8–3.0 ng/ml (2–4%).

**Figure 1.** Increased ear thickness (stars) in BALB/c mice after sensitization and challenge with TNCB. Values represent mean ± SEM of 8 mice. Time is hours following challenge.
Histologic Evaluation  Control ears exhibited intact mast cells (Fig 3A). The sensitized and challenged ears showed patchy (1+) degranulation between 1 h and 6 h (Fig 3B,C). Extensive degranulation (2+) was consistently observed only at 12 h. At 24 and 48 h, no significant degranulation of mast cells was noted (Fig 3D). Evaluation of mast cell numbers in the ears from mice which were sensitized and challenged showed a slight decrease at 24 h (data not shown), which was not statistically significant. Hypogranulated cells were seen in all the challenged tissues from immunized mice (Fig 3C), but were especially prominent at 48 h; this, however, was not statistically significant. Although some hypogranulated mast cells were observed in the irritant controls, active degranulation was not noted in any of the tissues examined.

DISCUSSION

Previous evidence suggesting a role for the mast cell in ACH can be summarized as follows. The areas in which ACH can be elicited following sensitization are preferentially populated with mast cells [5]. In mice, ACH is a reaction with early and late phases [6]. The early response, which is observed at 1–2 h, has been quantified by measuring tissue swelling [6] and leakage of $^{125}$I human serum albumin into the tissue [11]. The mast cell is believed to be responsible for this phenomenon inasmuch as prior depletion of mast cells with reserpine abolishes the response [4,5,12]. Animals treated with reserpine also fail to develop the late phase of ACH, suggesting an essential role for this cell [4]. Histologic examination of the ears has shown a decrease in the number of mast cells in the late stages of ACH [13,14]. Histamine also has been detected in increased concentrations in the urine of animals 24 h after the elicitation of ACH [15]. Finally, peritoneal mast cells from sensitized mice have been found to degranulate in vitro when exposed to the immunizing antigen [16].

Studies on strains of mice known to be deficient in mast cells ($W/W^b$, $W/W^s$ and $Sl/Sl^b$), however, have raised questions concerning the obligatory role of the mast cell in ACH. Based on radio metric assays of the cellular infiltrate, these mice have been shown to develop ACH reactions not significantly different from those of their littermate controls with normal numbers of mast cells [2,7,8]. In addition, studies utilizing reserpine must be re-evaluated given the fact that this agent has recently been shown to decrease cell-mediated immune reactions in vitro and in vivo [9].

In our study, the ear swelling response was found to be triphasic with peaks at 1.5, 8, and 24 h (Fig 1). Serum histamine levels

Figure 2. Change in serum histamine levels (stars) in BALB/c mice after sensitization and challenge with TNCB. Values represent mean ± SEM of 8 mice. Time is hours following challenge.

Figure 3. Histologic appearance of the ears of BALB/c mice. A, Control ear exhibiting intact mast cells (Giemsa x 132). B, Sensitized and challenged ear 1 h post challenge exhibiting mast cell degranulation (Giemsa x 330). C, Sensitized and challenged ear 4 h post challenge exhibiting mast cell degranulation and hypogranulated cell (arrow) (Giemsa x 330). D, Sensitized and challenged ear 24 h post challenge, exhibiting intact mast cells and infiltrating inflammatory cells (Giemsa x 330).
were elevated at 1–4, 12, and 48 h (Fig 2). The source of histamine was presumably the mast cell as suggested by the histologic findings (Fig 3). Although mouse platelets contain small amounts of histamine [17] and may have accounted for some of the histamine assayed, the increased levels are probably not due to platelet activation since both the unchallenged mice and the irritant controls failed to demonstrate increased levels. In addition, we have quantified histamine in platelet-rich and platelet-poor plasma from BALB/c mice and found no detectable differences in histamine levels between these plasma specimens and normal mouse serum (data not shown). In order to assess the possibility of histamine being derived from the formed elements of the blood, normal animals had plasma and formed elements assessed for histamine content. The formed elements contained a small percent of total blood histamine (2–4%), indicating that the majority of the histamine was tissue derived. Since mice have only a small number of basophil precursors in the bone marrow [18], it is unlikely that basophils accounted for the observed histamine elevation.

Askenase and coworkers have suggested that mast cell–derived vasoactive amines are responsible for the early response of ACH and allow the late response to develop by forming endothelial cell gaps which permit the effector cells to infiltrate the tissue [19]. Our results, while showing an early response in car swelling at 1.5 h and an increase in histamine levels between 1–4 h, also showed a response at 8–12 h (Figs 1, 2). Not only were the car swelling responses at 8 h and the histamine elevation at 12 h greater, but examination of challenged tissue showed extensive mast cell degranulation (+) at this time (Fig 3). If vasoactive amines are, in fact, important in ACH, this second peak would be of greater relevance since it more closely corresponds to the appearance of the cellular infiltrate seen maximally at 24 h [13, 14]. The presence of the third peak of histamine at 48 h, a time of decreased car swelling, suggests that histamine, which is known to exert a stimulatory effect on suppressor T lymphocytes, may also be involved in down-regulating the reaction in the late stages.

In a study by Mackenzie et al [11] during which the local extravasation of [131I]human serum albumin was examined after sensitization and challenge of mouse ears with oxazolone, maximum extravasation occurred between 40–50 min, 2–7 h, and 8–15 h. Some extravasation was also noted at 48 h. These time points are almost identical to the times at which we observed serum histamine elevations. Together, these data clearly indicate that mast cells, through their release of vasoactive amines and perhaps other mediators, alter the milieu of the challenged tissue at specific times during ACH, suggesting a modulating role for the cells in ACH. The mechanisms by which this occurs remain unresolved. There is evidence that IgE and other immunoglobulins are synthesized during sensitization by day 6 but not earlier [20, 21]. In the present study we chose to challenge at day 7, at the time when IgE would be expected to be present in order to permit its maximal expression. In addition, a reaction has been reported to occur in unsensitized mice by the infusion of antidiintrolphenol monoclonal IgE prior to challenge with dinitrofluorobenzene [22]. Since the reaction cannot, however, be transferred by serum from sensitized animals but can be transferred by lymph node cells, spleen cells, and peritoneal exudate cells [6, 23], a role for T cells has also been postulated. There is evidence suggesting that T cells indeed may produce factors that act on mast cells in an analogous fashion to IgE [24]. However, unlike IgE, these T-cell factors appear to release serotonin from nongranule storage sites with only moderate mast cell degranulation [25, 26]. Our results showing histologic evidence of mast cell degranulation and histamine release suggest that in this particular setting more than one mechanism of mast cell activation may be operative.

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