CA). Perinuclear staining was confirmed by counterstaining with propidium iodide.
16. Because most cell deaths (109 out of 131) occur 4 to 8 hours after fertilization (*9*), a heat shock was given to embryos at about 1 hour of development. Early embryos from *hsbcl-2* parents were collected from adults that had been cut open with a needle. The embryos were allowed to develop until early gastrulation (30 min at 20°C) and subjected to a heat shock (15 min for 33°C). The embryos were allowed to develop until the end of morphogenesis (7 to 9 hours at 20°C). Individual embryos were placed on an agar pad and photographed using Nomarski optics microscopy (*9*).
17. Only some embryos from *hslacZ* and *hsbcl-2* parents carry transgenic sequences, corresponding to the fraction of progeny inheriting an extrachromosomal array. In these cases, the *rol-6* marker gene was used to determine the genotype of each embryo; embryos that grew into adults with a roller phenotype or that segregated progeny with a roller phenotype were scored as transgenic. Some animals died due to the heat shock treatment, and the data from these animals were not included in this experiment.
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Neutrophil Recruitment by Tumor Necrosis Factor from Mast Cells in Immune Complex Peritonitis

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During generalized immune complex–induced inflammation of the peritoneal cavity, two peaks of tumor necrosis factor (TNF) were observed in the peritoneal exudate of normal mice. In mast cell–deficient mice, the first peak was undetected, and the second peak of TNF and neutrophil influx were significantly reduced. Antibody to TNF significantly inhibited neutrophil infiltration in normal but not in mast cell–deficient mice. Mast cell repletion of the latter normalized TNF, neutrophil mobilization, and the effect of the antibody to TNF. Thus, in vivo, mast cells produce the TNF that augments neutrophil emigration.

Leukocyte-endothelial cell interaction is a pivotal event in leukocyte emigration from the intravascular to the interstitial space during inflammation. TNF-α enhances neutrophil adhesion to endothelial cells in vitro. This is due, in part, to the expression of the adhesion proteins E-selectin (ELAM-1) and ICAM-1 by endothelial cells. The same cytokine also stimulates various cell types to release the neutrophil chemotactant interleukin-8 (IL-8). TNF-α also seems to be directly chemotactic to certain leukocytes, producing the up-regulation of integrin expression on the cell surface of leukocytes as well as the activation of these cells (*1*). Macrophages, neutrophils, T lymphocytes, natural killer (NK) cells, and mast cells can be stimulated to synthesize TNF-α (*1*). However, only mast cells store TNF-α in their granules. Activated mast cells, therefore, release TNF-α immediately from preformed stores and later release newly synthesized TNF (*2*). This suggests that TNF-α derived from mast cells may participate in the early phase of inflammation. Mast cell degranulation, TNF-α, and venular endothelial cell expression of E-selectin are correlated in human skin organ cultures exposed to morphine (*3*). Mast cells can also generate various other cytokines, including IL-1 (*4*). These cells are strategically located in high density around blood vessels, especially postcapillary venules, which are the principal sites of plasma flux and leukocyte transmigration into the interstitium. Mast cells are involved in neutrophil elicitation, plasma exudation, edema and fibrin deposition in reverse Arthus reaction [which is characterized by generalized antigen-antibody complex formation (*5–7*)] and antibody-mediated basement membrane injury (*8, 9*) in the skin, and neutrophil influx in thioglycollate- and immune complex–induced peritonitis (*10, 11*). Mast cells are also involved in inflammation induced by phorbol myristate acetate and substance P (*12, 13*) and in antigen-induced arthritis (*14*). The present study shows the significance of mast cell–generated TNF-α in the recruitment of neutrophils in immune complex–mediated inflammation.

Reverse passive Arthus reaction in the peritoneal cavity (intravenous injection of antigen and intraperitoneal injection of antibody) of mast cell–deficient $WBB6F_1$-W/W^v (W/W^v) mice and their congenic controls, $WBB6F_1$-+/+ (+/+), was chosen as the model of inflammation for the evaluation of the release and role of TNF-α, with mast cells as the possible source of TNF-α. Neutrophil accumulation was time-dependent and reached a maximum at 6 hours in both sets of mice (Fig. 1). However, the leukocyte influx was reduced in W/W^v mice, and in +/+ mice significant neutrophil infiltration was observed earlier (15 min) than in the W/W^v mice (30 min). Mast cells seemed to account for at least 40% of the neutrophil response. Correction of the mast cell deficiency of W/W^v mice

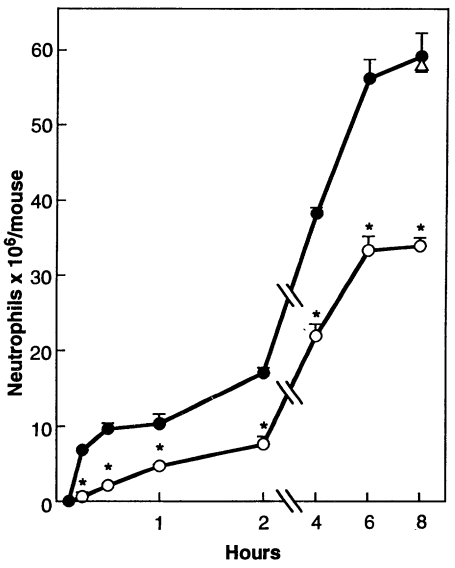

Fig. 1. Neutrophil influx in peritoneal reverse passive Arthus reaction in normal and mast cell–deficient mice. $WBB6F_1$-W/W^v (○), normal congenic control $WBB6F_1$-+/+ (●) (*21*), and mast cell–reconstituted W/W^v (△) (*11*) mice were anesthetized with pentobarbital (40 to 50 mg per kilogram of body weight, intraperitoneally) and intravenously injected with chicken ovalbumin (20 mg/kg). The mice were then immediately injected (intraperitoneally) with rabbit immunoglobulin G (IgG) to chicken ovalbumin (800 μg per mouse) or phosphate-buffered saline (PBS). At the times indicated after challenge, mice were asphyxiated with CO_2, and their peritoneal cavity lavaged with 1 ml of PBS containing 0.1% bovine serum albumin. The protocol used in the care of the mice was in accordance with institutional guidelines. The peritoneal fluid was centrifuged at 200*g* for 10 min. Neutrophil influx was evaluated by measurement of myeloperoxidase (MPO) activity colorimetrically in the cell lysate (*22*). The number of neutrophils present was determined from a standard curve of MPO activity of known numbers of neutrophils (obtained from peritoneal exudate after 6 hours of reverse Arthus reaction). MPO activity in peritoneal cells of PBS-treated mice was equivalent to 0.7×10^6 neutrophils. Data are expressed as mean ± SEM (n = 3 to 6); *P < 0.01, comparison of +/+ and W/W^v mice at the respective time points, as determined by the Student's t test.

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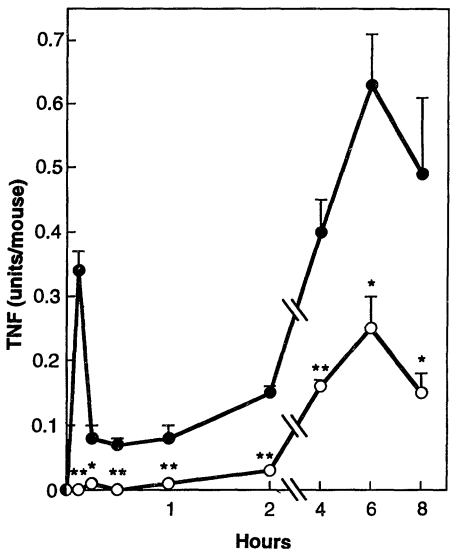

Fig. 2. TNF accumulation in the peritoneal cavity. Cell-free supernatants of the lavage fluid, obtained from +/+ (●) and W/W^v (○) mice treated as in Fig. 1, were assayed for TNF by a cytotoxicity assay (*23*), and the units of TNF present were determined by comparison to the activity of TNF-α standard. The zero time point represents measurements from PBS-treated mice. Data are represented as mean ± SEM (n = 3 to 6); $^*P < 0.05$ and $^{**}P < 0.005$, comparison of +/+ and W/W^v mice at the respective time points, as determined by the Student's t test.

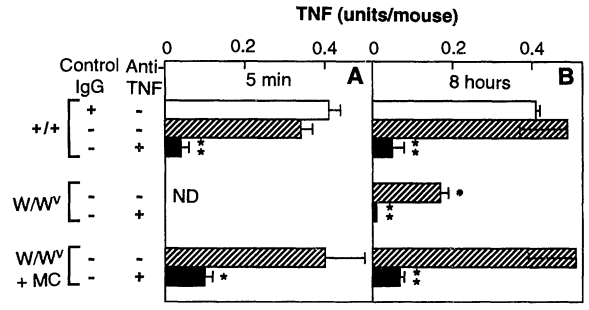

Fig. 3. Confirmation of the presence of TNF. Reverse passive Arthus reaction was induced as in Fig. 1. TNF activity was determined 5 min (**A**) and 8 hours (**B**) after challenge as in Fig. 2, in the presence or absence of neutralizing hamster MAb to mouse TNF or control hamster IgG (MAb to mouse IL-1β or to mouse IL-2) (*24*) (50 μg/ml). Data are expressed as mean ± SEM (n = 3 to 6); $^*P < 0.05$ and $^{**}P < 0.002$, comparison to samples without MAb to TNF; $^•P < 0.05$, comparison of W/W^v to either +/+ or W/W^v + MC (W/W^v reconstituted with mast cells) mice (no MAb to TNF in the assay); ND, not detected. P values were determined by the Student's t test.

normalized neutrophil recruitment (Fig. 1). Adoptive transfer of mast cells corrects only the mast cell deficiency and no other abnormality of W/W^v mice (*11*, *15*). These findings confirm our previous observation that mast cells promote neutrophil elicitation during inflammation induced by immune complexes (*5*, *8*, *11*). Mast cells seem to be recruited early in the reaction by complement (*16*), which is activated during reverse Arthus reaction (*17*).

We evaluated TNF-α release by measuring TNF activity in the peritoneal fluid. In +/+ mice, two peaks of cytotoxic activity were observed (Fig. 2). The initial peak (5 min) declined by 15 min, but TNF activity remained elevated. A second peak of greater magnitude than the first one occurred between 4 to 8 hours after challenge. In the exudate from W/W^v mice, the early peak was absent, and the late peak was reduced by 60% (Fig. 2). Mast cell reconstitution of W/W^v mice restored the cytotoxic activity at 5 min and 8 hours after challenge, as compared to the activity in +/+ mice (Fig. 3). The cytotoxic factor in the peritoneal exudates of +/+, W/W^v, and mast cell–repleted W/W^v mice was confirmed immunologically to be TNF (Fig. 3). Thus, the early peak of TNF in vivo originated from mast cells and is probably due to the release of the granule-stored cytokine.

The second peak probably consists of newly synthesized TNF, the source of which is not known. Cells such as resident macrophages and recruited neutrophils in the peritoneal cavity could contribute to the late peak. The peritoneal macrophages are probably not the major source of this TNF, because TNF was substantially reduced in W/W^v mice; these mice have normal macrophages and monocytes. The macrophage count in the peritoneal fluid of W/W^v mice was equivalent to that in +/+ mice (+/+, 4.06 × 10^6; W/W^v, 4.48 × 10^6, $P > 0.05$). Monocytes are not recruited to the site of inflammation until later (>16 to 24 hours). The lower TNF activity of the late peak in W/W^v mice was probably not directly related to the mast cell deficiency in these mice because mast cells constitute <5% of the total resident peritoneal cells (and even less after neutrophil influx). The simultaneous increase in TNF and neutrophils, the concomitant reduction in both parameters as a result of mast cell deficiency, and the augmentation of these parameters with mast cell reconstitution suggest that neutrophils may be the major source of the cytokine of the second peak. The greater TNF activity in +/+ than in W/W^v mice may be due to the higher number of neutrophils present. Mast cells may, therefore, indirectly influence the peritoneal TNF activity as a result of their ability to enhance neutrophil influx.

The interpretation that neutrophils constitute the major contributors of the second TNF peak is further suggested by the effect of the leukotriene synthesis inhibitor A-63162 (*18*). This drug produced a comparable suppression of neutrophils and TNF activity at 8 hours in +/+ mice (Fig. 4). As in our previous observations (*5*, *8*, *19*), A-63162 had no effect in W/W^v mice. This suggests that mast cells are responsible for the generation of the chemotactic leukotrienes that mobilize neutrophils, which in turn release the TNF. A-63162 does not suppress mast cell degranulation (5) and did not alter the early (5 min) TNF peak (Fig. 4B). Mast cell repletion of W/W^v mice restored the effect of A-63162 on both the

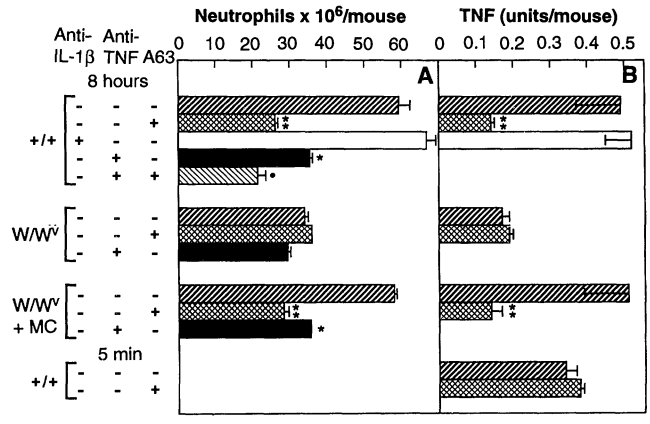

Fig. 4. Effect of TNF, IL-1β, and leukotrienes on neutrophil elicitation and TNF activity. W/W^v, +/+, and W/W^v + MC (W/W^v reconstituted with mast cells) mice were intraperitoneally injected with neutralizing MAb to TNF or IL-1β (250 μg per mouse, 18 hours before challenge) (*24*) or leukotriene synthesis inhibitor A-63162 (*19*). This dose of A-63162 effectively blocked leukotriene formation by 90% in both +/+ and W/W^v mice (*19*). At the times indicated after the initiation of the Arthus reaction, neutrophil influx (**A**) was evaluated as in Fig. 1 and TNF activity (**B**) as in Fig. 2. Control mice that did not receive any antibody or A-63162 were given PBS intraperitoneally or 0.2% methylenclulose orally. Data are expressed as mean ± SEM (n = 3 to 6); $^*P < 0.05$ and $^{**}P < 0.001$, comparison to challenged +/+ mice with placebo pretreatment; $^•P < 0.002$, comparison to challenged +/+ mice with placebo pretreatment; and $P < 0.02$, comparison to +/+ mice treated with MAb to TNF. P values were determined by the Student's t test.

neutrophil response and peritoneal TNF activity (Fig. 4).

We studied the functional significance of mast cell–derived TNF on neutrophil mobilization by first treating mice with a neutralizing monoclonal antibody (MAb) to TNF. At 8 hours after stimulation, the concentration of neutrophils in +/+ mice, but not W/W^v mice, treated with this antibody had decreased by 40% (Fig. 4A). Local repletion of W/W^v mice with mast cells restored the effect of MAb to TNF (Fig. 4). This observation implies that TNF-α is involved in the elicitation of neutrophils and that mast cells are a critical source of this TNF-α. The location of mast cells near the microvasculature may be crucial for this effect. The increase in the expression of inflammation correlates with the migration of adoptively transferred mast cells into the tissue of W/W^v mice over time (*10, 11*). In the skin of W/W^v mice, a large number of the intradermally injected mast cells were later found around small blood vessels (5). The W/W^v mice generated a significant amount of TNF at 4 to 8 hours, comparable to that in the first peak of +/+ mice (5 min); nevertheless, MAb to TNF did not attenuate the neutrophil response in W/W^v mice (Fig. 4). This observation suggests that the second TNF peak is not critical for the recruitment of neutrophils. Only the preformed TNF-α released from mast cells early in the reaction produces neutrophil infiltration. MAb to TNF, in combination with A63162, further reduced neutrophil influx. However, the difference was significant only as compared to treatment with MAb to TNF and not to treatment with A63162 alone (Fig. 4). It appears that both the chemotactic leukotriene and TNF-α, which may cause the expression of E-selectin, are required simultaneously for neutrophil emigration. The present findings in mice are probably also important for inflammation in humans. Stimulation of mast cells in human skin organ culture resulted in the expression of E-selectin and neutrophil influx that was TNF-α–dependent (*3*).

Adhesion of neutrophils to endothelial cells in vitro is also enhanced by IL-1β (*20*). However, the neutralizing MAb to IL-1β did not block the neutrophil response (Fig. 4). This indicates that IL-1β does not have a detectable role in neutrophil influx in the present model. This confirms a similar observation made in skin organ cultures. TNF-α, but not IL-1β, from degranulated mast cells seemed to be the main cytokine responsible for inducing E-selectin expression on the venular endothelium (*3*). Mast cells and other cells release IL-1 after stimulation. However, unlike TNF, IL-1 is not known to be stored. It is newly synthesized and released only a few hours after challenge. Therefore, the time of its release would probably coincide with the second TNF peak.

The experiments described in the present study show that mast cells participate in vivo in neutrophil recruitment by releasing stored TNF early during the immune complex–induced inflammatory reaction. The late release of this cytokine appears to be produced mainly by the activity of neutrophils. Mast cells have a significant impact on peritoneal TNF concentrations not only by releasing TNF but also by their ability to enhance neutrophil mobilization.

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