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Anopheles Mosquito Bites Activate Cutaneous Mast Cells Leading to a Local Inflammatory Response and Lymph Node Hyperplasia

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When Anopheles mosquitoes probe the skin for blood feeding, they inject saliva in dermal tissue. Mosquito saliva is known to exert various biological activities, but its perception by the immune system and its role in parasite transmission remain poorly understood. In the present study, we report on the cellular changes occurring in the mouse skin and draining lymph nodes after an Anopheles stephensi mosquito bite. We show that mosquito bites induce dermal mast cell degranulation, leading to fluid extravasation and neutrophil influx. This inflammatory response does not occur in mast cell-deficient W/Wv mice, unless these are reconstituted specifically with mast cells. Mast cell activation caused by A. stephensi mosquito bites is followed by hyperplasia of the draining lymph node due to the accumulation of CD3⁺, B220⁺, CD11b⁺, and CD11c⁺ leukocytes. The T cell enrichment of the draining lymph nodes results from their sequestration from the circulation rather than local proliferation. These data demonstrate that mosquito bites and very likely saliva rapidly trigger the immune system, emphasizing the critical contribution of peripheral mast cells in inducing T cell and dendritic cell recruitment within draining lymph nodes, a prerequisite for the elicitation of T and B lymphocyte priming. The Journal of Immunology, 2005, 174: 3932–3940.

The adaptation of the hematophagous arthropods (mosquitoes, sandflies, ticks, and so forth) to blood feeding is an essential parameter of host-vector-parasite interactions well exploited by the parasite. Indeed, the insect mouthparts, penetrating the skin like a syringe, pierces a mammalian’s first line of defense against pathogens/parasites and gives them instant access to the host’s subepithelial microenvironment. Host skin defenses, in which the ultimate goal is to prevent a successful blood meal, consist of blood leakage, hemostasis, pain and itch responses, and immune effector mechanisms. The pharmacological properties of saliva (1) may represent the insect’s answer to these mechanisms. They include anticoagulant, antiplatelet, vasodilatory, and anti-inflammatory/immunosuppressive activities (1, 2), which contribute to overcome the host defense mechanisms.

The parasite microorganisms have also taken benefit from these pharmacological properties. There is now compelling evidence that vectors are not only syringes injecting parasites and that co-injected blood-feeding insect saliva has profound effects on parasite transmission (3, 4). Indeed, Plasmodium berghei sporozoites delivered into mice through mosquito bites are more infectious than salivary gland-extracted sporozoites injected i.v., suggesting a facilitating role of saliva in parasite transmission (5). Other examples include the parasite Leishmania major, which infectivity is increased considerably by coinjection of a salivary gland extract from sandfly vectors Phlebotomus papatasi (4) and Lutzomyia longipalpis (6) and the Thogoto arbovirus, which benefits from tick saliva (7).

Host tissue reactions to salivary components following the insect probing and blood-feeding vary from small papules to large pruritic swellings depending on mosquito species. They are mild in unsensitized persons and may become more pronounced if allergic sensitization against saliva occurs. Such mechanisms include type I (immediate, IgE-dependent) and type IV (delayed-type hypersensitivity (DTH)⁴, cell-mediated) hypersensitivities (4, 8) and indicate that some components of mosquito saliva are allergenic. Interestingly, a sandfly saliva component-targeted DTH response has protective effects with regard to L. major-driven pathogenic processes, suggesting that vaccination against saliva may be of protective value against insect-borne pathogens and parasites (8).

Mast cells are potent sentinels of the innate immune system (9). Besides well-known IgE-dependent mast cell degranulation during allergic reactions, mast cells also degranulate in the absence of IgE Abs to nonimmunologic mast cell activators such as polycationic compounds, including 48/80, substance P, bradykinin, mastoparan, and polyethyleneimine (9–13), and in response to several pathogens. Mediators released by mast cells cause the wheal and flare reaction, edema and itching symptoms of the allergic reaction resulting from vasodilation, enhancement of vascular permeability, and stimulation of sensory nerves (14). Certain mediators such as TNF-α and MIP-2/IL-8 have been shown to be chemotactic for neutrophils, which then enter the inflamed tissue (15), and this...
represents a major mechanism for bacterial clearance (16). Mast cells are also endowed with immunomodulatory properties (15) exerting both stimulatory (17) and suppressive effects (18) on the adaptive immune response. In the dermis, mast cells and dendritic cells often found intimately connected (19) could influence each other as exemplified by dendritic cell phenotypic changes after mast cell degranulation (20).

We hypothesized that mosquito saliva might exert immunomodulatory effects within a host exposed to mosquito bites through induction of mast cell degranulation, which in turn affects dendritic cell migration and function. Such a mechanism could be of importance for malaria parasite transmission. We report herein that Anopheles bites induce skin mast cell degranulation, resulting in a local fluid extravasation and recruitment of neutrophils at the site of mosquito bite. Anopheles stephensi saliva directly induces isolated connective-tissue type but not mucosal-type mast cells to degranulate in vitro in the absence of IgE Abs. Of note, mosquito bites also induce dendritic cell migration to draining lymph nodes and lymph node hyperplasia without neutrophil influx. Finally, mosquito bites cause substantial increase of TNF-α concentrations within draining lymph nodes, but this increase does not seem to be essential for mosquito bite-induced lymph node hypertrophy.

Materials and Methods

Animals

Female BALB/c, C57BL/6, and DBA/2 mice 6–8 wk old were purchased from R. Janvier (Laval, France). Mast cell-sufficient (WWB6F1−/−) and control-congenic mast cell-deficient (WWB6F1−/−WW/WW) mice were purchased from The Jackson Laboratory and were raised in our animal facility. All animal care and experimentation was conducted in accord with the Pasteur Institute animal care and use committee guidelines. A. stephensi (sda 50 strain; CEPIA Laboratory, Pasteur Institute) was maintained at 26°C with 75% relative humidity under a 12-h photoperiod. Adult mosquitoes were provided a 10% sugar solution, and females were blood-fed on anesthetized rabbits biweekly. Larvae were fed on Friskies Cat Chow.

Reagents and Abs

Murine recombinant stem cell factor (SCF) was purchased from Strathmann Biotec. Mouse rIL-3 was purchased from Biosys. Recombinant TGF-β1 and IL-9 were purchased from BioSource International. The MP6-XT22 rat anti-mouse TNF-α hybridoma (DNAX) was obtained through the American Type Culture Collection. The Ab was purified from culture supernatant by using protein G immobilized on Sepharose 4B beads fast flow (Sigma-Aldrich). Normal rat IgG of the same isotype (Jackson Immunoresearch Laboratories) was used as a control. FITC-labeled anti-mouse Ab directed against CD11c, CD3, CD11b, and B220 were purchased from BD PharMingen. FITC-labeled rat IgG2a and IgG2b control isotypes were purchased from BD PharMingen. PE-labeled mouse IgG2a and FITC-labeled hamster IgG control isotype were purchased from Tebu. PKH26 and compound 48/80 were purchased from Sigma-Aldrich.

Cultures of mouse and human mast cells

Bone marrow-derived mouse mast cells (BMMC) were prepared as described by Razzin et al. (21) and modified by us (22). After 3 wk of culture using RPMI 1640 medium (BioWhitaker) supplemented with 10% heat-inactivated FCS (Boehringer Mannheim) and 3 U/ml rIL-3, the cells were harvested and consisted of 98% pure mast cell populations as assessed by toluidine blue staining and c-Kit and FceRI expression. Connective tissue mouse mast cells (CTMC) were obtained according to the procedure described elsewhere (23) with the following slight modifications. Instead of using 3T3 fibroblasts as a feeder-layer, BMMC were cocultured with syngenic fibroblasts from the posterior eye cavity for 3 wk in the presence of 10 U/ml rIL-3 and 10 U/ml rSCF. Medium (complete RPMI 1640 medium supplemented with 10% FCS) was replaced every week. In contrast to BMMC developed in IL-3, these CTMC were found to secrete β-hexosaminidase in response to Gi-coupled polyacetylated compounds such as compound 48/80 and substance P (Sigma-Aldrich), indicating that they were of connective tissue type (23).

Mucosal-type mast cells were obtained, according to the procedure described by Miller et al. (24), by culturing cells with 5 ng/ml IL-9, 1 ng/ml IL-3, 50 ng/ml SCF, and 1 ng/ml TGF-β1. Cells were fed every 2–3 days by changing the half-volume of original culture medium by fresh medium. Cells were used after 15 days of culture and consisted of 95% mast cells, as shown by toluidine blue staining. The novel SCF-dependent human mast cell line, designated LAD-2, established from bone marrow aspirates from a patient with mast cell sarcoma/leukemia was a kind gift from Dr. Dean Metcalfe (National Institute of Allergy and Infectious Diseases, Bethesda, MD) and was maintained in the presence of 100 ng/ml recombinant human SCF (Amgen). It expresses FcεRI and contains intracytoplasmic histamine, tryptase, and chymase. The LAD-2 cell line releases β-hexosaminidase following FcεRI or FcγRI aggregation (25).

Mast cell reconstitution of W/Wv mice

W/Wv mice were reconstituted locally with mast cells by injecting intradermally in the back skin 2 × 106 cultured CTMC 48 h before exposure to mosquito bites. In some experiments, W/Wv mice were reconstituted systemically with mast cells by i.v. injection of 2 × 106 cultured BMMC 6 wk before exposure to A. stephensi mosquito bites.

Exposure of mice to mosquito bites

Mice were anesthetized by i.p. injection of ketamine (600 mg/kg) and xylazine (20 mg/kg) and were placed on top of mosquito cages to allow for bites through the mesh. Mosquito bites were focused on a site of ventral or dorsal skin by limiting an area of ~2 cm2 using paper tape. To allow exposure to saliva with minimal bleeding of animals (maximum 40 bites/mouse/session), mosquito feeding was disrupted every 2–3 min, and Anopheles, which have taken blood meal, were counted systematically. All mice were naive before exposure to mosquito bites.

Assessment of fluid extravasation

Anesthetized mice received an injection of Evans blue dye (1% in PBS; 50 μl/mouse) into the retro-orbital vein immediately before exposing their shaved back skin for 1 h to Anopheles bites. Animals were sacrificed 2 h later, and the back skin was taken for examination and photography of the internal skin side. Bites can be easily detected by the presence of 0.5- to 1-mm diameter hemorrhagic spots in the hypodermis.

Tissue processing for histological analysis

Mice were euthanized, and a piece of dorsal skin was gently flattened onto a piece of thick paper to avoid curling and fixed in 4% paraformaldehyde for 24 h. Fixed tissues were embedded in paraffin, and serial sections (4-μm thick) were stained with 2% toluidine blue for 2 min. For each sample, measurements were made on five separate histological sections, and the number of mast cells per centimeter length of tissue was determined by microscopic examination (×250 magnification). Measurements were obtained from 10–15 W/Wv mice or mast-cell reconstituted W/Wv mice. For analysis of leukocyte infiltration, the entire sample was cut to take a 4-μm section every 100 μm. These sections were stained with H&E and examined with a microscope. Infiltrates were classified arbitrarily as strong, mild, or absent. Additional histochemical analysis to reveal chloroacetate esterase (CAE) activity was performed in Dr. Steven’s laboratory (Department of Medicine, Brigham and Women’s Hospital, Boston, MA, and Department of Medicine, Harvard Medical School, Boston, MA) by using freshly fixed paraformaldehyde-embedded in resin and cut and stained according to the procedure described by Friend et al. (26). Both mast cells and neutrophils are positive for CAE activity but can be distinguished easily by the nucleus shape and by the fact that mast cell CAE activity is much stronger and located in the granules. Slides were read by two observers blinded to the origin of the samples.

Mast cell degranulation assay

Female A. stephensi mosquitoes (6–10 days old) were allowed to bite through an artificial membrane (Stretched Parafilm; American Can Company) mounted on a glass minifeeder maintained at 37°C by warm water circulation (27). To characterize saliva bioactivity, mast cells were incorporated into the minifeeders at the concentration of 106/ml in Tyrode buffer and were exposed to mosquito bites (100 female mosquitoes) for 2 h. As controls, minifeeders containing the same mast cell preparations were kept out of reach of mosquitoes. Cells were harvested, and after centrifugation, supernatants were analyzed for their content in β-hexosaminidase as described previously (28). All results are expressed as a percentage of total β-hexosaminidase in the cells after correction for spontaneous release in unstimulated cultures (net release).
Lymph node cell preparation and flow cytometry analysis

Inguinal lymph nodes from (+/+), W/W', and mast cell reconstituted W/W mice, which have received mosquito bites on the back, were taken, homogenized, and a single-cell suspension was obtained by filtration over a 100-μm nylon cell strainer. After three washes in PBS containing 1% FCS, cells were incubated with anti-FcγRI/II/III Ab (clone 2.4G2, BD Pharmingen) 20 min before addition of fluorochrome-coupled, Ag-specific mAbs. Flow cytometric analysis was performed on a FACSCalibur flow cytometer (BD Biosciences).

Treatment with anti-TNF-α Ab

To assess the role of TNF-α in mosquito bite-induced lymph node hyperplasia, 250 μg of rat anti-mouse TNF-α or an isotype-matched control were injected twice into the peritoneal cavity of naive WBB6F1-KitW/KitW-v mice 24 and 3 h before exposure of mice to mosquito bites or to a challenge with compound 48/80 (Sigma-Aldrich). In some experiments, to trigger polyclonal as well as inguinal lymph nodes, this compound was injected intradermally at the dose of 1.2 mg/kg body weight in 25 μl in the footpad and in the ventral and dorsal skin. Likewise, A. stephensi were allowed to bite at similar skin locations as compound 48/80. Control skin sites and footpads received sterile PBS. Inguinal and popliteal lymph nodes were harvested 48 h later, and cell counts were performed. The capacity of the anti-TNF-α Ab to neutralize the biological activity of TNF-α was assessed by using the WEHI-164 cell clone cytotoxicity assay (1 μg/ml of the Ab was found to completely neutralize the cytotoxic activity of 0.5 ng/ml TNF-α; data not shown).

Detection of TNF-α by ELISA

Inguinal and popliteal lymph nodes were harvested 1, 3, and 6 h after mosquito bites or after challenge with compound 48/80, put into 100 μl of PBS, and frozen and thawed five times as described previously (29). After homogenization, supernatants were analyzed for their TNF-α content by ELISA, according to the manufacturer’s instructions (R&D Systems). Because the presence of TNF-α was found to be optimal after 3 h of stimulus, only results related to this time point were presented.

Assessment of in vivo lymphocyte proliferation

WBB6F1-KitW/KitW-v mice were injected i.v. twice at 4-h intervals with BrdU (1 mg/mouse; Sigma-Aldrich). To ensure continuous labeling, BrdU was added at a concentration of 0.8 mg/ml to drinking water for 48 h, which was freshly prepared daily and protected from light. Immediately after the first injection of BrdU, mice were exposed or not to mosquito bites for 15 min (average of 30 bites). Mice were sacrificed 48 h later, their inguinal lymph nodes harvested, and cells were counted and prepared for labeling with anti-BrdU Abs. Briefly, cells were surface labeled with FITC-conjugated anti-CD3 Ab. The cells were then washed, fixed, and permeabilized using paraformaldehyde solution containing 0.005% Tween 20. Fixed cells were incubated with DNase for 1 h at 37°C and then washed and stained with Alexa-labeled anti-BrdU Ab for FACS analysis.

T lymphocyte labeling with PKH26

Single-cell suspensions were obtained from spleens of WBB6F1-KitW/KitW-v mice, and T cells were positively selected using anti-CD90-coated magnetic beads (StemCell Technologies). After several washings, this T cell population (96–99% T cells as assessed by flow cytometry using an Ab to CD3) was labeled with PKH26, according to the manufacturer’s instructions.

Labeled T cells (5 × 10⁶) were injected i.v. into mice, followed immediately by exposure or not to mosquito bites. Draining lymph nodes were collected, cells harvested, and the percentage of labeled T cells present in the preparations was assessed by flow cytometry.

Statistical analysis

The paired two-tailed Student t test was used with p = 0.05 taken as the level of significance.

Results

Mosquito bites cause mast cell-dependent vascular permeability in the mouse skin

Mosquito bites usually cause local cutaneous inflammatory reactions with small papules, erythema, and pruritic swelling. We made the hypothesis that, in addition to direct anticoagulant effects, mosquito saliva contains components with mast cell-degranulating activity contributing to this reaction. Local mast cell activation classically observed during the passive cutaneous anaphylaxis reaction results in the release of vasoactive amines, which induce vascular permeability visualized by extravasation of Evans blue dye. The same procedure was performed to characterize skin reactions upon mosquito bites. Normal WBB6F1-KitW/KitW-v mice received Evans blue dye by i.v. injection and then were exposed to mosquito bites for 1 h. As shown in Fig. 1A, blue stains can be seen around hemorrhagic spot lesions caused by mosquito bites. This reaction was dependent on the presence of mast cells in the skin because mast cell-deficient WBB6F1-KitW/KitW-v (W/W') mice showed less or no reaction (Fig. 1B), which was restored in mast cell-reconstituted W/W' mice (Fig. 1C). To demonstrate that there was no bias in preferential biting avidity of mosquitoes for +/+ and W/W' mice, the quantity of bites based on the number of mosquitoes that have effectively taken their blood meal was determined, and no significant difference was found between the two types of mice (data not shown).

To provide microscopic evidence of mast cell activation, histologic preparations were made from biopsies taken at the site of mosquito bites. Fig. 2 shows that in contrast to control sites, mast cell degranulation occurred at the bitten skin sites as indicated by the release of mast cell granules revealed by toluidine blue staining and their chloroacetate esterase activity. Up to 30% of mast cells (average 22%) were degranulated at the site of mosquito bite, whereas no sign of degranulation could be observed in control skin samples. Mast cell degranulation was observed mostly in the s.c. tissue where hemorrhage occurred, suggesting that the insect saliva was injected at this skin layer rich in veins and arteries.

FIGURE 1. Astephensi bite induces a mast-cell dependent vasopermeabilization in mouse skin. Normal WBB6F1-KitW/KitW-v (n = 10), mast cell-deficient WBB6F1-KitW/KitW-v (n = 10), or mast cell-reconstituted W/W' (W/W'-R) mice were anesthetized and received an injection of Evans blue dye into the retro-orbital vein before exposing their back skin for 1 h to Astephensi bites. Animals were sacrificed 2 h later, and size and intensity of Evans blue spots on the internal face of the skin from +/+ (A), W/W' (B), and W/W'-R (C) mice were estimated visually. Shown is a representative example of three experiments. Bar: 1 cm.
Anopheles saliva directly triggers mast cell degranulation

During a mosquito bite, cutaneous mast cell degranulation may be triggered directly by saliva or can result from the effects of intermediate factors generated from the tissue environment. To determine whether salivary components directly induce mast cell degranulation, mouse mast cells were incorporated into glass minifeeders equipped with stretched parafilm mimicking skin (27) and were exposed to mosquito bites. This procedure allows freshly produced saliva to gain access to mast cells and therefore ensures the bioactivity of salivary components. After 2 h of exposure, the mast cell suspension was centrifuged, and the supernatant was assessed for the presence of β-hexosaminidase activity. As a control, mast cells were incubated in feeders placed out of reach of mosquitoes. As shown in Fig. 3, connective tissue mast cells generated from either DBA/2 or C57BL/6 mice peritoneal cavity were specifically activated upon mosquito bites. Similarly, the recently described SCF-dependent tryptase/chymase-positive (connective type) human mast cell line LAD-2 could also be induced to release β-hexosaminidase activity upon mosquito bites. In contrast, exposure of IL-3-derived BMMC (immature mast cells) (data not shown) or mucosal-type mast cells (bone marrow derived) generated using a mixture of cytokines consisting of IL-3, SCF, IL-9, and TGF-β1 (24) failed to respond to mosquito bites. These data suggest that salivary components directly induce degranulation of human and mouse mast cells of connective but not of mucosal type without the need for other cell types present in the skin.

Mast cell-dependent leukocyte infiltrate in the skin upon mosquito bite

To investigate the histologic changes occurring following mast cell degranulation induced by mosquito bites, +/+ and W/W mice were exposed to Anopheles females for 1 h, and skin biopsies were taken 3 h later. Hemorrhage was always found at the limit between the dermis and s.c. tissue, suggesting that a mosquito’s mouthparts pierced skin to that depth where vessels are larger than the capillaries and veinules found in the upper dermis. Skin biopsies centered on the hemorrhage revealed that an inflammatory infiltrate represented by granulocytes could be found at the center of that hemorrhagic zone (Fig. 4A). Revelation of chloroacetate

![Image](http://www.jimmunol.org/)

FIGURE 2. Degranulation of mouse dermal mast cells at the site of A. stephensi bite. Normal WBB6F1−/− mice (n = 6) were exposed (B and D) or not (A and C) to Anopheles bites on the back skin for 1 h. Skin samples centered to the bite site were taken 2 h later and were prepared for histology. Resting and degranulated mast cells were visualized by staining skin sample preparations using toluidine blue (A and B) or by the revelation of chloroacetate esterase activity (C and D).

![Image](http://www.jimmunol.org/)

FIGURE 3. A. stephensi saliva directly induces mast cell degranulation in vitro. Mast cells (10^7/ml) from the peritoneal cavity (connective type) or bone marrow-derived mast cells (mucosal type) of DBA/2 or C57BL/6 mice were placed in glass minifeeders and were exposed to mosquito bites for 2 h, and the release of β-hexosaminidase was assessed using a colorimetric assay of the enzymatic activity. Mast cells placed in a minifeeder out of the reach of mosquitoes were used as a negative control. The human mast cell line LAD-2 was examined in the same way. Shown are the mean ± SD of three to five independent experiments.

![Image](http://www.jimmunol.org/)

FIGURE 4. The inflammatory infiltrate in bitten skin sites is mediated by mast cells. Normal WBB6F1−/− (n = 6) mice were exposed to Anopheles bites as in Fig. 2, and skin samples were taken 2 h later for histology. Sections showing leukocyte infiltrates could be evidenced by H&E staining (A) and by the presence of chloroacetate esterase activity, which indicates that these were essentially neutrophils (B). To analyze the mast cell contribution to this process, three types of mice (normal +/+ (n = 6), mast cell-deficient W/W (n = 8), and mast cell-deficient W/W6 (n = 6) mice reconstituted with connective type mast cells (W/W-R)) were exposed similarly to Anopheles bites, and skin samples were taken. Toluidine blue-stained sections were used to estimate mast cell density in samples from control (unbitten) skin (C). A proportion of skin samples showing strong (dark gray bars) or weak (light gray bars) inflammatory infiltrates as assessed by H&E staining in the skin of different mouse groups exposed to mosquito bites (D).
esterase activity demonstrated that the predominant cells were neutrophils (Fig. 4B), otherwise completely absent from control tissues (data not shown). This reaction was dependent on the presence of skin mast cells because no leukocyte infiltration was observed in the skin of W/Wv mice (Fig. 4D). To provide a quantitative evaluation of these histological changes, skin biopsies were classified arbitrarily into strong, mild, or no leukocyte infiltrate after microscopic observation. Fig. 4, C and D, shows that a lack of mast cells in W/Wv mice correlated well with the absence of strong infiltrates because such infiltrates occurred in ~73% of skin biopsies from +/- mice, whereas none was present in W/Wv mice. More than 80% of W/Wv mice had no infiltrate at all, and only mild leukocyte infiltrates could be observed in others. To demonstrate that the lack of infiltrate was really due to the lack of mast cells and not to any unidentified deficiency of the W/Wv strain, such mice were reconstituted by injection of mast cells. Reconstitution indeed restored the ability to develop local inflammatory infiltrates (Fig. 4D).

Anopheles bites induce lymph node hyperplasia and leukocyte influx

Mast cell activation mediated by mosquito bites may alter the migratory properties of dermal dendritic cells and modify the cellular content of draining lymph nodes. To investigate whether mosquito bites actually induce such changes, +/- mice were exposed to Anopheles bites on the back skin 1–3 days before collection of the inguinal lymph nodes. Mice not exposed to mosquito bites served as controls. A significant swelling of lymph nodes could be seen both at macroscopic observation and cell counts (Fig. 5A). FACS analysis of lymph node cell suspensions revealed that mosquito bites significantly increased the CD11c+ dendritic cell number and that the percentage reached a plateau at 48 h after the mosquito bites (Fig. 5B). Additional analysis of lymph node cellularity 48 h after mosquito bites showed that an increase in leukocyte recruitment was not selective because distinct cell populations, including CD3+ (T cells), B220+ (B cells), CD11c+, and CD11b+ cells, were all found to be enhanced significantly (Fig. 5, D, E, F, and G, respectively). Microscopic examination of preparations colored with May-Grünwald-Giemsa staining revealed that neutrophils were absent; therefore, CD11b+ cells were macrophages. To determine whether this lymph node enlargement resulted from the proliferation of resident cells or from a cellular influx, mice were treated immediately after the mosquito bites with BrdU, which incorporates into proliferating cells. Analysis of lymph node cell reactivity to a fluorescent anti-BrdU Abs revealed that the fraction of proliferating cells was small (between 1 and 3% of total cells) and could not account for lymph node enlargement (Fig. 6A). On the contrary, the fraction of proliferating thymocytes from the same mice tested as positive control was high (from 50 to 70%).

To confirm that mosquito bites induce an influx of circulating lymphocytes, mice were injected with PKH-26-labeled T cells and were immediately exposed or not to mosquito bites. Fig. 6B shows that 48 h after mosquito bites, a significantly higher number of PKH-26+ T cells were found sequestered in draining lymph nodes from bitten mice as compared with control mice.

Because TNF-α was shown recently to be a potent mediator of lymph node hypertrophy (29), we examined its contribution to mosquito bite-induced lymph node hyperplasia by neutralizing anti-TNF-α Abs. First, we examined whether mosquito bites induce TNF-α in draining lymph nodes and whether neutralizing anti-TNF-α Ab is able to prevent TNF-α accumulation within lymph nodes. As shown in Fig. 7A, increased levels of TNF-α were found in lymph nodes 3 h after mosquito bites or after a challenge with the mast cell secretagogue compound 48/80. TNF-α could be detected as early as after 1 h (data not shown), suggesting that it was preformed in mast cells, the only cell type able to store it. Accumulation of TNF-α in lymph nodes was completely precluded when mice were pretreated with anti-TNF-α Ab. Next, we sought to investigate the contribution of TNF-α to the lymph node hypertrophy induced by mosquito bites. Ppopliteal and inguinal lymph node hypertrophy, as assessed by increased leukocyte number, was measured in mice untreated or treated with control IgG or with neutralizing anti-TNF-α Ab 24 and 3 h before exposure to mosquito bites. As depicted in Fig. 7B, the increase in

![FIGURE 5.](http://www.jimmunol.org/) A. stephensi bites induce hyperplasia of draining lymph nodes. WBB6F1-/- mice (n = 5) were exposed or not to Anopheles bites on the back skin and were sacrificed at indicated times, after which, inguinal lymph nodes were collected, and single-cell suspensions were prepared. Time course analysis of total leukocytes (A) and CD11c+ cells (B) in draining lymph nodes of mice exposed or not to mosquito bites. Analysis of draining lymph node populations 48 h after mosquito bites was performed: total leukocytes (C), CD3+ (D), B220+ (E), CD11c+ (F), and CD11b+ (G) cells.

![FIGURE 6.](http://www.jimmunol.org/) Mosquito bites induce T cell recruitment in draining lymph nodes. The number of CD3+ T cell blasts labeled in vivo with BrdU was determined in lymph nodes (A) and in thymuses (B) from WBB6F1-/- mice (n = 5) exposed or not to mosquito bites (A). Total number of PKH-26-labeled T cells sequestered in draining lymph nodes from mice (n = 6) exposed or not to mosquito bites (B).
stituted W/Wv (W/Wv-R) mice were exposed or not to mosquito saliva. Inguinal and popliteal lymph nodes from WBB6F1−/− mice untreated or treated 24 and 3 h prior to stimulation with anti-TNF-α or isotype control Abs. Data are expressed as mean + SD from six mice per group. The number of cells per lymph node, and the values represent the mean + SD from six mice per group.

FIGURE 7. TNF-α is not essential for lymph node hypertrophy induced by mosquito bites. A, TNF-α protein was measured by ELISA in the lymph nodes 3 h after mosquito bites or injection of compound 48/80 in the footpads of WBB6F1−/− mice untreated or treated 24 and 3 h prior to stimulation with anti-TNF-α or isotype control Abs. Data are expressed as mean + SD (six mice per group) of TNF-α concentration in lymph node homogenate. B, Inguinal and popliteal lymph nodes from WBB6F1−/− control naive mice and mice, which received two injections of either neutralizing anti-TNF-α or isotype control Abs 24 and 3 h before exposure to mosquito bites, were harvested 48 h later, and total cells were counted. Data representative of two independent experiments are presented as number of cells per lymph node, and the values represent the mean + SD from six mice per group.

The observation that the cutaneous inflammatory response induced by mosquito bites was expressed fully if mast cells were present in the tissue led us to explore the effect of mast cells on the leukocyte influx into the draining lymph nodes induced by mosquito bites. Normal (+/+), mast cell-deficient (W/W−), and mast cell-reconstituted W/W− (W/W−-R) mice were exposed or not to mosquito bites, and 48 h later, draining lymph nodes were harvested and examined for their cellular content. As shown in Fig. 8, the number of total leukocytes (Fig. 8A) and CD11c+ cells (Fig. 8B) induced by mosquito bites was increased significantly in lymph nodes from +/+ mice and W/W−-R mice but not in W/W− mice. These data demonstrate that mast cells are essential for the occurrence of leukocyte influx in the skin but also for the sequestration of leukocytes, including CD11c+ dendritic cells in lymph nodes induced by mosquito saliva.

Discussion

We report here that A. stephensi mosquito bites result in a local IgE-independent degranulation of skin mast cells as assessed by a rapid dermal inflammation and the hyperplasia of the draining lymph nodes. This provides a mechanism whereby mosquito bites may shape the immune response to microorganisms present in their saliva and inoculated during the blood meal. The sites of mosquito bites are characterized by fluid extravasation and rapid infiltration with polymuclear neutrophils. Our data demonstrate that this local reaction results from mast cell activation in the absence of IgE, as demonstrated by the fact that mast cells are degranulated, and 2) it is not observed in mast cell-deficient animals unless they are reconstituted with mast cells. Mast cells are known to induce such local reaction by rapidly releasing several mediators, including granule-associated histamine, tryptase, TNF-α, MIP-2, as well as eicosanoids, including leukotrienes. A parallel can be made with a previous work showing that increased plasma extravasation was induced by mosquito bites in repeatedly immunized mice with Aedes albopictus salivary gland extracts (30). However, this effect was ascribed to a IgE- and IgG1-mediated specific immune response. In an earlier report (31), the intradermal injections with A. albopictus salivary gland extracts in humans showed that a high m.w. fraction elicited an immediate and a delayed response similar to a bite reaction. In our system, the mast cell-dependent inflammatory response at skin sites exposed to A. stephensi mosquito bites occurs in naive mice. This indicates that some components in Anopheles saliva have the capacity to directly trigger mast cell activation in the absence of salivary-induced specific Abs. This does not preclude the possibility that repeated exposures to Anopheles mosquito bites may induce sensitizing-specific IgE Abs, which may then exacerbate the inflammatory response. The mosquito bite also induced cellular infiltrate and hyperplasia of the draining lymph nodes. These alterations consist of an increase of the number of various leukocyte lineages, including T lymphocytes, B lymphocytes, dendritic cells, and monocytes/macrophages. In vivo measurements of proliferating cells using anti-BrdU mAbs have established that, although a marginal homeostatic proportion of proliferating cells was detected in lymph nodes, the majority of leukocytes was very likely...
recruited from the blood vascular compartment or lymphatic vessels. Therefore, the presence of mast cells at the skin site of the mosquito bite not only controls the local granulocyte influx but also promotes leukocyte (but not neutrophils) influx in lymph nodes.

Recently, it has been reported that peripheral mast cells play a critical role, through the TNF-α they produce, in regulating the hypertrophy of draining lymph nodes during a bacterial infection or following activation by the mast cell secretagogue 48/80 (29). Our data support these findings in that saliva-induced activation of dermal mast cells similarly induces lymph node swelling via the recruitment of T and B lymphocytes, dendritic cells, and monocytes/macrophages. Similarly to the observation made by McLachlan et al. (29), in situ activation of skin mast cells upon mosquito bites led to the accumulation of TNF-α within draining lymph nodes. Detection of TNF-α as early as 1 h after mosquito bites (data not shown) strengthens the idea that this TNF-α was preformed in mast cells granules. However, in contrast to their findings, in vivo TNF-α neutralization using the same TNF-α-specific Ab (at even higher doses) failed to inhibit lymph node hypertrophy elicited by mosquito bites. The TNF-α-neutralizing capacity of the Ab used was demonstrated in vitro (data not shown), as well as in vivo by the reduction of TNF-α in lymph nodes close to baseline levels in mice treated with the Ab before exposure to mosquito bites or to a challenge with compound 48/80 (Fig. 7A). Therefore, our data indicate that, although mosquito bites and bacterial infection (the McLachlan model) both result in rapid accumulation of TNF-α and infiltrating cells in draining lymph nodes, they differ in that this cytokine does not seem to be essential for mosquito bite-induced lymph node hypertrophy. In addition to TNF-α, mast cells are a source of numerous mediators and chemokines (32), which might play the key role in our system. Among them, the MCP-1 is known to promote leukocytes entry into lymph nodes (33). Also, the CXC chemokine MIP-2 has been implicated in neutrophil recruitment in models of bacterial host defense and T cell-mediated DTH (16, 34). It was found abundantly in and around mast cells in a mouse model of contact hypersensitivity reaction, was absent in mast cell-deficient mice (34), and was involved in the migration of dendritic cells (35). Therefore, MCP-1 and MIP-2 are good candidates for the elicitation of both local and distal inflammatory responses induced by mosquito bites. It is now well established that the resident dendritic cells of an inflamed tissue are induced to mature and to migrate to draining lymph nodes via afferent lymphatics. Therefore, we think that the dendritic cells observed in our experiments originate from the site of bites. This view is supported by several reports showing that IgE-dependent mast cell activation promotes the migration of dendritic cells to the draining lymph nodes (36).

Among additional questions that need to be addressed, it is important to investigate how mosquito bite-induced mast cell activation affects Ag-specific immune responses. An immunosuppressive effect mediated by mast cells has been reported previously in a contact hypersensitivity model in which UVB light induced mast cell degranulation (18, 37). During the sensitization phase of DTH response, dendritic cells capture the Ag, migrate to draining lymph nodes, and undergo a maturation process required for the activation of naive T cells. We speculate that mosquito saliva induces the release of a particular set of inflammatory mediators by activated mast cells that may affect the maturation of adjacent dendritic cells, which fail to ultimately elicit fully activated effector T cells. It is known that the ability of dendritic cells to direct the development of naive T cells into Th1, Th2, or regulatory T cells is largely dependent upon the signals that they receive in the peripheral tissues at the time of Ag capture. We are currently investigating the molecular mechanisms underlying this mast cell-mediated regulation of the immune response.

In addition to high-affinity IgE receptors, mast cells can be activated through various receptors reacting with endogenous (neuropeptides and anaphylatoxins) or exogenous stimulants. Indeed, mosquito bites activate mast cells in vivo in the absence of IgE or IgG Abs directed against salivary components. Moreover, in vitro experiments revealed that the saliva of A. stephensi directly triggers mediator release from connective tissue-type mouse and human mast cells but not from mucosal-type mast cells. Mast cells may also be activated by a large number of microorganism-derived molecules through TLR or CD48 surface molecules. Human mast cells express TLR 1, 2, 6, and 9 (38), whereas mouse mast cells express TLR 1, 2, 4, 6, and 8 (39). It is possible that some salivary components could be considered as the long-known signature of blood-feeding insects and could be recognized by pattern recognition receptors expressed by sentinel mast cells. Our observation that connective-type but not mucosal-type mast cells respond to saliva suggests that such receptors would be preferentially expressed by the former, a possible evolutionary adaptation of host skin to react to vector saliva. Although mosquito saliva directly activates mast cells, the possibility that intermediate tissue factors would participate to mast cell activation in situ during naturally occurring mosquito bites cannot be excluded. Such factors could contribute to amplify the response in vivo. Mast cell histamine release can be triggered by nonimmunological stimuli such as compound 48/80, substance P, mastoparan, bradykinin, and spermine, which all directly activate Go proteins (10). In this regard, the specificity responsiveness of connective tissue-type but not mucosal-type mast cells to saliva would fit with a selective distribution of Gi molecules or distinct susceptibility of Gi molecules among these two types of mast cells. Current experiments are undertaken to identify the component(s) of the saliva mediating mast cell activation and to determine whether they act via a known receptor expressed by mast cells.

It is tempting to speculate that the mosquito carrying Plasmodium sporozoites in its saliva would deposit these very motile zoites, not directly in the blood vessel but in the hemorrhagic pocket from where they can then escape toward the blood flow. This is supported by the observation that sporozoites remain ~5–10 min in the tissue before reaching the systemic circulation (40). Therefore, because of the rapid response of mast cells, it is likely that sporozoites encounter degranulated mast cells and their products. Whether this inflammatory environment influences sporozoite infectivity is not known and is currently being investigated in our laboratory.

In the case of a mosquito infected by Plasmodium falciparum, the saliva-induced mast cell degranulation reported here could act together with the translationally controlled tumor protein, a homologue of the mammalian histamine-releasing factor secreted by this parasite (41). Translationally controlled tumor proteins such as histamine-releasing factor have been shown to cause the release of histamine and promote IL-8 secretion in human eosinophils (42). The skin response to various mosquito species probably involves a combination of direct and indirect effects of saliva activities. For example, saliva from the mosquito Culex pipiens contains histamine, and the benefit is thought to reside in the vasorelaxant properties of histamine, which facilitates the blood meal collection. The mechanical lesion might also cause some reactions from the tissue. Saliva from various insects do not seem to exert the same effects and therefore do not contain the same sets of active molecules. Indeed, saliva from the bloodsucking hemipteran Rhodnius prolixus contains antihistamine factors (1).
Our observation that mosquito bites cause neutrophil infiltration only in mast cell-sufficient mice clearly demonstrates that neutrophils are attracted at the site of the mosquito bite by mast cell products and could contribute to the host defense against the parasites delivered by the mosquito. Whether neutrophils have a capacity to fight Plasmodium sporozoites is not known. A high m.w. glycoprotein from A. stephensi saliva endowed with a direct neutrophil chemotactic activity has been observed in vitro (43). Possibly due to differences in terms of dose and freshness of saliva mediators, our work in vivo does not support these observations because mosquito bites failed to induce neutrophil infiltration in mast cell-deficient mice and rather emphasizes a critical role of mast cells in the accumulation of neutrophils at the site of mosquito bite.

During inflammatory responses, lymph nodes become the meeting point between entrapped circulating lymphocytes and Ag-presenting dendritic cells that have sampled the Ag in the periphery. The adaptive immune response results from this encounter and therefore benefits in terms of rapidity and intensity from the role played by mast cells in cell migration. It will be interesting to examine whether mast cells also influence the nature of the immune response. In individuals sensitized against mosquito saliva and in malaria patients who develop parasite-specific IgE (44), these Abs may increase mast cell responsiveness and potentiate their role as sentinels. Our goal is now to understand whether saliva facilitates Plasmodium transmission and to better define the contribution of skin mast cells in Plasmodium establishment.

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