

Urticaria Neonatorum: Accumulation of tryptase-expressing mast cells in the skin lesions of newborns with Erythema Toxicum

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Erythema Toxicum, a rash frequently present in the healthy newborn infant is an innate, immune response to the first commensal micro flora. Flushing and urtication are seen in this manifestation suggesting mast cell (MC) activation and MC derived mediator release. It has recently become evident that MCs participate in the protective, innate immune response against microbes also by secreting products toxic to pathogens such as cathelicidin peptide antibiotics. We hypothesized that MCs contribute to the process of inflammation in Erythema Toxicum and that skin MCs of human newborns express the cathelicidin peptide antibiotic LL-37. Skin sections were immunostained for MC tryptase. Double immunofluorescence was performed by staining LL-37 in combination with tryptase. We studied ultra structure of skin MCs with transmission (TEM) and immunoelectron microscopy (IEM). Seven infants with and six infants without the rash, as well as three adults were included. We found numerous tryptase-expressing MCs recruited around the hair follicles in the lesions of Erythema Toxicum. TEM analysis of MCs exhibited signs of degranulation in the lesion. Neither skin MCs from newborns nor adults did express LL-37 as judged by confocal and IEM. MCs participate in the inflammatory responses of Erythema Toxicum by taking an active part in the immune system of the hair follicle. However, their immunological activity is not linked to the expression of the cathelicidin antimicrobial peptide LL-37. A pivotal role of MCs in the innate, inflammatory response at the site of pathogen invasion during the critical time of perinatal colonization is suggested.

Annika Nelson¹, Ann-Kristin Ulfgren², Josefine Edner¹, Berit Ståbi³, Hjalmar Brismar^{1,4}, Kjell Hultenby⁵ and Giovanna Marchini¹

¹Department of Woman and Child Health, Neonatal Unit, Karolinska Institutet, ²Department of Medicine, Rheumatology Unit, Karolinska Institutet, ³Division of Obstetrics and Gynaecology, Karolinska Institutet, ⁴Department of Cell Physics, Royal Institute of Technology, ⁵Division of Clinical Research Centre, Department of Laboratory Medicine, Karolinska University Hospital in Huddinge, Karolinska Institutet, Stockholm, Sweden

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Giovanna Marchini, Neonatal Unit, Astrid Lindgren Children's Hospital, FRH-lab, C4:U1 Karolinska University Hospital Solna, S-171 76 Stockholm, Sweden
Tel.: 46 8 51773817
Fax: 46 8 323048
E-mail: giovanna.marchini@karolinska.se

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Erythema Toxicum is a transient rash present to various degrees in most healthy newborn infants during the first days after birth (1). It is characterized by an accumulation of leukocytes, particularly eosinophils in dermal lesions (2), and recent reports link its pathogenesis to the penetration of the first commensal micro flora into the skin followed by an innate, immune response (3–6). The lesions may consist of scattered urticaria-like spots, especially during the early phase of the rash (Fig. 1a,b). In fact, the rash was previously also known as urticaria neonatorum (7), based on the

belief that it was an allergic manifestation (8, 9). Clinical symptoms of urtication combined with the fact that antihistamine given to newborns with erythema toxicum shortened the course (10) implements an involvement of mast cells (MCs) in this manifestation. Recent research enfances the critical and multifaceted role of MCs in the innate immune defence against infectious organisms. MCs interact with the vasculature and participate in the recruitment of circulating leukocytes with bactericidal properties, crucial for a full response during acute infection (reviews 11, 12).

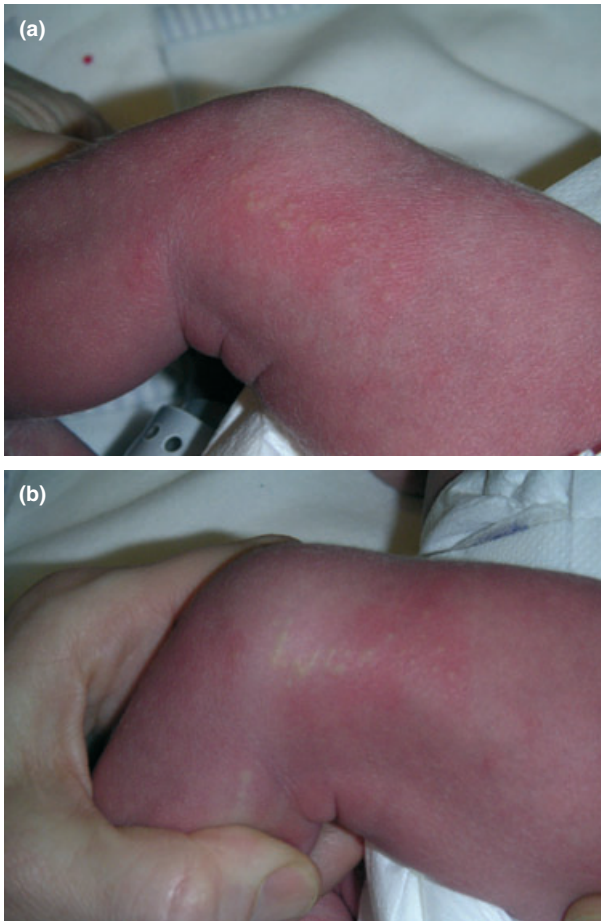


Fig. 1. Maculo-papular eruption in a 1-day-old infant (a), note blanching where skin is being stretched (b).

Human skin MCs also secrete the cathelicidin peptide antibiotic LL-37 as indicated by *in vitro* studies on human mastocytoma (13). Cathelicidins comprise a family of antimicrobial peptides that have been identified in epithelial tissues and some myeloid cells of humans and animals (14). As antimicrobials, cathelicidins have activity against a wide range of bacteria, fungi, viruses and protozoa (review 15). Additionally, cathelicidins chemoattract immune cells, activate diverse types of leukocytes and epithelial cells (review 15) by that contributing to mobilize the immune system and link innate and adaptive immunity of the host. About 30 different cathelicidins have been described in mammals, but LL37 is the only gene-encoded cathelicidin present in humans, so far known (16). The expression of LL37 is upregulated during inflammatory skin disorders not only in adults (17) but also in newborns with Erythema Toxicum (5).

Here, we seek (i) to study a possible participation of MCs in the lesions of Erythema Toxicum and (ii) to investigate if skin MCs of

human newborns express the human cathelicidin peptide antibiotic LL-37, and (iii) to examine the ultra structure of skin MCs in newborns, including signs of activation.

Materials and methods

Study population

The ethics committee of Karolinska Hospital, Stockholm, Sweden, approved the study and all parents gave informed consent for their infant to participate in the study. All infants were born at term, after an uncomplicated pregnancy and delivery and all had an uneventful neonatal period. The infants were 1 day old at the time the skin biopsy specimen was taken. They all had adequate birth weight for gestational age and were exclusively breastfed on demand. Punch biopsies of 3 mm were obtained after local anesthesia, as previously described (4). Thirteen infants were included, eight girls and five boys, 12 of white skin color and one of black skin color; seven had a generalized urticaria-like rash with maculae-papules. All biopsies were obtained from lesions on the lower extremity. As control material, biopsy specimens of normal skin was obtained from corresponding skin regions of six unaffected infants and from three healthy adults.

Methods

Immunohistochemistry. Biopsy specimens were cryostat sectioned and fixed in ice-cold acetone-methanol (1:1) and pure acetone. Endogenous peroxidase activity was blocked with hydrogen peroxide in phosphate buffered saline (PBS). All subsequently steps were carried out in the PBS bovine serum albumin (PBS-BSA) 0.05% buffer. Immunohistochemical analyses were performed staining MC tryptase with a mouse anti-human monoclonal antibody (Chemicon international, Temecula, CA, USA). The slides were incubated overnight at room temperature in a humid chamber with the primary tryptase antibody at a concentration of 1.5 µg/ml. As a positive tryptase control, we used human synovial MCs. Negative control staining was performed by excluding the primary antibody. The slides were washed and incubated with normal horse serum, followed by incubation with the biotinylated secondary antibody horse anti-mouse IgG, diluted in normal horse serum. Finally, the slides were incubated with an avidin-biotin-horse-radish peroxidase complex (ABC-stand, Vector Lab, Inc., Burlingame, CA, USA). The brown color reaction was developed by 0.5 mg/ml

diaminobenzidine (DAB-kit, Vector Lab Inc.). Blue counterstaining was performed in Mayer's 10% hematoxylin and the slides were mounted in Kaiser's glycerin-gelatin. Six tissue sections for each case were examined, including two sections that were used as negative controls. The estimation of the number of tryptase-expressing cells was based on a semi-quantitative scale where +, few positive cells, i.e., <20 cells at one site with magnification $\times 200$, and ++, numerous cells. All sections were manually analyzed by an Axioplan Universal microscope (Zeiss, Jena, Germany). Two different observers analyzed the sections; they were blinded to the groups.

Double immunofluorescent analyses were performed with LL-37 rabbit anti-human polyclonal antibody (a kind gift of Birgitta Agerberth, Medical biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden) in combination with tryptase mouse anti-human monoclonal antibody (Chemicon International). Cryosectioned samples, fixed as previously described, were blocked with 4% goat serum in PBS with 1% BSA and 0.1% saponin by incubation for 1 h at room temperature in a humid chamber. Sections were incubated overnight at +4°C with LL-37 primary antibody at a concentration of 0.8 $\mu\text{g}/\text{ml}$, and tryptase primary antibody at a concentration of 2.0 $\mu\text{g}/\text{ml}$, both diluted in blocking buffer. After rinsing, the slides were incubated for 1 h at room temperature with the secondary fluorescent antibodies; goat anti-mouse Alexa 488 (Molecular Probes, Eugene, OR, USA) for tryptase, and goat anti-rabbit Alexa 546 (Molecular Probes) for LL-37. As a final step, the slides were washed with PBS containing 1% BSA and 0.1% saponin and mounted with Vectashield Hard-set (Vector Lab, Inc.). Specimens were recorded with a Leica TCP SP confocal microscope, using a 40 $\times$ /1.25 NA objective. Alexa 488 was excited at 488 nm and detected at 492–530 nm. Alexa 546 was excited at 543 nm and detected at 550–650 nm. Controls for auto fluorescence and unspecific binding were performed by excluding the primary antibodies against tryptase and LL-37. As positive control for LL-37, staining of keratinocytes was performed.

Transmission electron microscopy (TEM)

Two punch-biopsies; one from an infant with and one from an infant without the rash were obtained and processed for TEM analysis as previously described (3). Biopsies were fixed in 2% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M sodium cacodylate buffer containing

0.1 M sucrose and 3 mM CaCl_2 , pH 7.4 at room temperature for 30 min followed by 24 h at 4°C. Specimens were rinsed in 0.15 M sodium cacodylate buffer containing 3 mM CaCl_2 , pH 7.4, postfixed in 2% osmium tetroxide in 0.07 M sodium cacodylate buffer containing 1.5 mM CaCl_2 , pH 7.4 at 4°C for 2 h, dehydrated in ethanol followed by acetone and embedded in LX-112 (Ladd, Burlington, VT, USA). Semithin sections were cut and stained with toluidinblue and used for light microscopic analysis. Ultrathin section of approximately 40–50 nm were cut and contrasted with uranyl acetate followed by lead citrate and examined in a Tecnai 10/Leo 906 transmission electron microscope at 80 kV. Digital images were captured by a Mega View III digital camera (Soft Imaging System, GmbH, Münster, Germany).

Immunoelectron microscopy (IEM)

Biopsy from one infant with the rash was obtained and fixed in 3% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer. Samples were then infiltrated into 2.3 M of sucrose and frozen in liquid nitrogen. Sectioning was performed at -95°C . Grids were placed directly on drops of 0.15 M NaCl containing 20 mM glycine followed by incubation in 2% BSA (Sigma fraction V) and 2% Gelatin (IGSS quality, Amersham Biosciences UK limited, Buckinghamshire, England) in 0.1 M phosphate buffer to block non-specific binding. Sections were then incubated with the LL 37 primary antibody (a kind gift of Birgitta Agerberth, Medical biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden) diluted 1:200 in 0.1 M of phosphate buffer containing 0.1% BSA and 0.1% Gelatin over night in a humidified chamber at room temperature. The sections were thoroughly washed in the same buffer and bound antibodies were detected with protein A coated with 10 nm gold (Amersham Biosciences UK limited) at a final dilution of 1:100. Sections were rinsed in buffer and fixed in 2% glutaraldehyde and contrasted with 0.1% uranyl acetate and embedded in 2% methylcellulose and examined in a Tecnai 10 (Eindhoven, The Netherlands) at 80 kV (18).

Results

Immunohistochemistry

In a biopsy of 3 mm, we found that there was only one hair follicle affected in Erythema Toxicum, exceptionally two. There was a focal, dense

accumulation of MCs in the dermis surrounding the hair follicle in the sections from lesions of Erythema Toxicum. A diffuse extracellular staining of the MCs, but also distinct intracellular tryptase staining of MCs was seen (Fig. 2a,b). MCs were also present in sections of controls, but to a much more limited extent and randomly scattered throughout the whole dermal layer (Fig. 2c). The number of tryptase-positive cells/site in controls was 18 ± 3 MCs (mean $\pm$ SD) at a magnification of $\times 200$, $n = 8$ (five infants + three adults). For details, see Table 1.

Double immunofluorescent staining of LL-37 in combination with tryptase

No co-location of LL-37 to the skin MC marker tryptase was found, neither in skin sections from human newborns (Fig. 3a) nor adults (Fig. 3b–f).

TEM

Examination of sections from two newborns, one with the rash and one without, identified MCs with typical morphology scattered in the dermal layer. The cell surface was covered with fine microvilli. Their cytoplasm was filled with dense, osmophilic secretory granules and few organelles. In the sections from the infant with the rash, numerous granules were more electron-lucent, especially at the cell periphery. Signs of granule extrusion were seen. The formed cavities contained altered granules. In some sections MCs also contained phagosomes with material surrounded by a distinct membrane structure (Fig. 4). Control sections contained a lower number of MCs and granules having a more uniformly dense, osmophilic structure, with no so-called altered granules, compared to sections from the lesion. No phagosomes were found in these MCs (data not shown). A total number of 20 MCs in sections from a control-infant and 50 MCs in sections from an infant with Erythema Toxicum, was analyzed.

IEM

Specific labeling for antibodies against LL-37 was found abundantly in the cytoplasm of both keratinocytes and neutrophils but not in MCs, identified by morphology (data not shown).

Discussion

We found a focal accumulation of tryptase-expressing MCs associated with the hair follicles

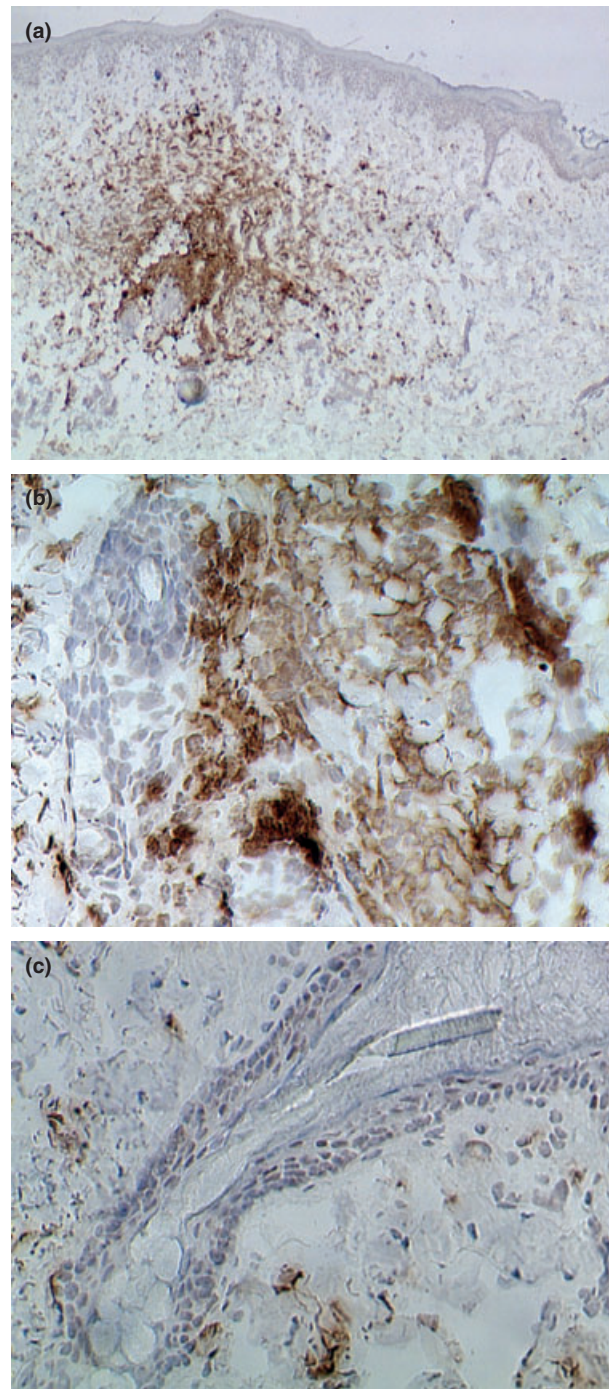


Fig. 2. Immunohistochemical staining with anti-tryptase, of sections from (a) a lesion of Erythema Toxicum showing the massive recruitment of mast cells in the dermis surrounding a hair follicle ($\times 100$), and (b) details showing intra/extracellular tryptase-staining near a hair follicle ($\times 250$), and (c) from a control-infant, with mast cells scattered in the dermis near a non-inflamed hair follicle ($\times 250$).

in the lesions of Erythema Toxicum; these cells also presented signs of degranulation, indicating cell activation, and some cells also contained phagosomes. The human cathelicidin peptide

Table 1. Scores for immunohistochemical staining of mast cell tryptase in biopsy-sections from 1-day-old infants with the rash of Erythema Toxicum, and from matched controls and healthy adults

	Infant 1	Infant 2	Infant 3	Infant 4	Infant 5	Adult 1	Adult 2	Adult 3
<i>Erythema Toxicum lesions</i>								
Tryptase positive cells								
Dermis	++	++	++	++	++			
<i>Non-inflamed skin</i>								
Tryptase positive cells								
Dermis	+	+	+	+	+	+	+	+

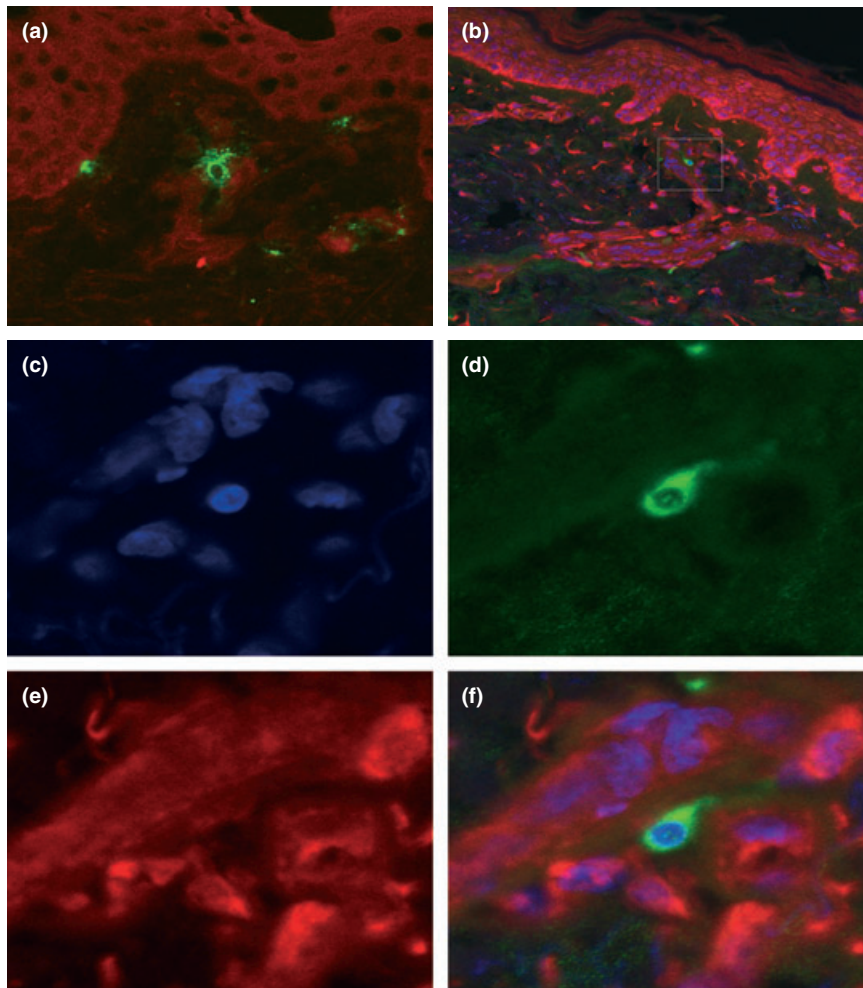


Fig. 3. Confocal imaging of (a) infant showing fluorescent staining of LL-37 seen in the whole keratinocyte layer, red color reaction. No colocation is shown with mast cell tryptase, green color reaction. In adult skin (b) LL-37 is expressed in the cytoplasm of the whole keratinocyte layer as well as in other immune cells, red color reaction. Distinct tryptase positive cells are seen as green color reaction, and nuclei counterstained with DAPI as blue. The tryptase positive cells are all lacking LL-37 signal. Detail of (b) is seen as nuclei counterstained with DAPI (c), MC stained with tryptase (d), LL-37 found abundantly in the cytoplasm of other immune cells (e), and (f) where all color reactions are found merged.

antibiotic LL-37 was not expressed in skin MCs of newborns or adults.

Mast cells originate from hemopoietic bone marrow precursors, circulate in the blood as immature progenitors, and complete their differentiation in the mucosal and connective tissues, including the skin where they are found in close proximity not only to nerves, vessels but also to hair follicles (review 11). Cutaneous mastocytosis (19) as well as Erythema Toxicum (3–6), may occur all over the skin but not on the palms and soles, areas lacking hair follicles, indicating a pivotal role for MCs in the hair follicle immune system.

Mast cells are highly granulated cells, a sign that Paul Erlich in 1879 interpreted as being ‘well fed’, Mastzellen (20). Their preformed granule-associated mediators such as tryptase (an enzyme unique to all human MC granules), histamine, cationic proteins and metabolites of the arachidonic acid cascade have effects on cellular recruitment and vascular permeability (review 11). Both MCs and eosinophils are found in frequent numbers at the gastro-intestinal barrier (21). They often co-locate in diseases such as asthma, parasitic disease and inflammatory bowel disease (reviews 11, 21). Here, we show

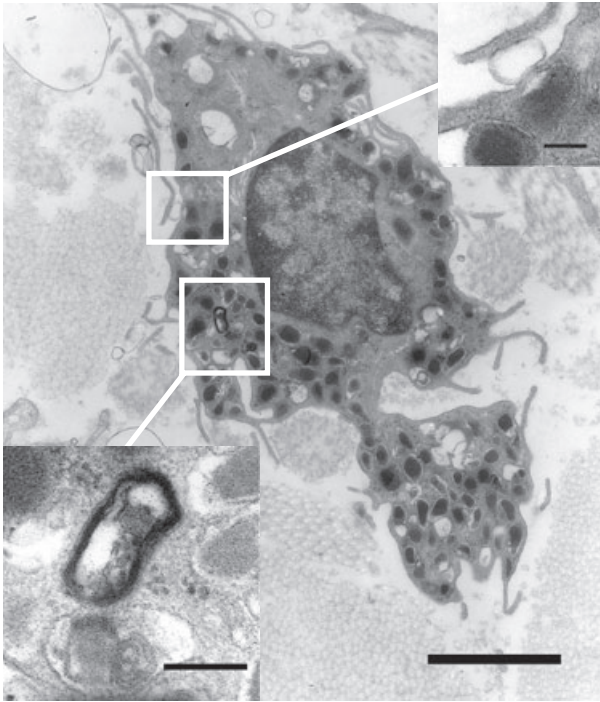


Fig. 4. Transmission electron microscopy (TEM) micrographs showing a mast cell with typical morphology, in the lesion of Erythema Toxicum (bar = 2 μ m). Note the fine microvilli on the cell surface and the cytoplasm with electron lucent granulae and clear signs of degranulation, especially at the periphery with fusions between cell- and perigranular membrane. Granules on their way out of the cell was seen (bar = 0.2 μ m). Note also the inclusion material surrounded by a distinct membrane structure, contained into a phagosomes (bar = 0.2 μ m).

that this is also the case in Erythema Toxicum. Their functional role in this context is unknown, but it seems likely that MC mediators may induce a variety of behavioral responses by neighboring cells in the micro-environmental location of Erythema Toxicum.

Mast cells may have developed as important effector cells in immune responses that are associated with T_H2 and IgE, because in the context of infections with helminth parasites, such effector functions can confer significant benefit to the host (review 22). From an evolutionary point of view it seems reasonable to assume that the newborn has a preparedness to resist the challenge of the broadest spectrum possible of commensal and/or pathological microbes, including that of helminths and other parasites, and not be limited to those of the 'artificially germ-free' modern world. This would be in line with the finding that human neonatal CD4-expressing T cells respond to a parasite antigen (23). The immune reaction of Erythema Toxicum, which we have now shown also includes MCs, may thus reflect a primordial,

stereotyped immune response to the commensal colonization of the human newborn. The activation of MCs in the newborn may also be connected to the polarized T_H2 immune response, typical for the newborn (24) and for children with allergic diseases (25). Recently, it has also been shown that MCs of the newborn have unique functions compared with their adult counterparts because of different gene profiles (26).

Structural changes of MC degranulation as we observed by TEM, were seen in the sections from the lesions of Erythema Toxicum. Degranulation was particularly evident at the periphery of the cell, in the granules immediately under the cell membrane. The granular substance underwent gradual loosening, revealing a finely, filamentous, more electron-lucent inner structure. Close apposition and fusion of the cell membranes and the perigranular membrane, with resulting cavity formation were seen. Similar electron microscopic findings are seen in degranulated human pulmonary MCs (27) and in rat MCs (28).

Mast cells recognize pathogens indirect via complement receptors or Fc-receptors (review 11) but they also have the capability to mediate direct recognition of pathogens by Toll-like receptors (29, 30) and mannose-binding proteins (31). By using MC deficient and reconstituted mice, it was shown that MCs are crucial for the survival in connection with bacterial infections (32). MC deficient mice were also up to 20-fold less efficient at clearing enterobacteria than MC-sufficient mice (33). These findings reflect a critical role of MCs in host defence and natural immunity. The presence of phagosomes into recruited MCs in Erythema Toxicum may reflect that a direct phagocytosis of microbes has occurred (Fig. 4). Similar phagosomes were previously reported in epithelial cells of the hair follicle as well as in near-located macrophages and eosinophils in Erythema Toxicum (3). Therefore, it seems reasonable to deduce that an induction of MC activity is initiated by a recognition and response to commensal skin microbes penetrated through the hair follicle.

We found no co-location of the cathelicidin peptide antibiotic LL-37 to skin mast cells of newborns or adults, neither with immunofluorescent analysis nor with IEM. These findings may reflect that under normal conditions cathelicidins are not active as antimicrobials in human skin mast cells and that cathelicidins do not participate in the mast cell innate response in Erythema Toxicum.

In summary, we have found that MCs participate in the complex inflammatory response of Erythema Toxicum. However, their immuno-

logical activity does not seem to be linked to the expression of LL-37. The MC-mediated activity could be especially important in the modulation of immune responses to microbes in naive individuals, as the case of newborn infants. Further studies addressing possible MC-eosinophil interactions at birth are needed.

Acknowledgment

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