Bullous Allergic Hypersensitivity to Bed Bug Bites Mediated by IgE against Salivary Nitrophorin

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In Central Europe, bites from the common bed bug (*Cimex lectularius*) are nowadays rather uncommon. Nevertheless, infestations are sometimes observed in old framehouses and by immigration due to international travel and migration. The clinical picture of bug bites substantially varies between individuals, depending upon previous exposure and the degree of an immune response. The host immune response and potential protein antigens present in the saliva of *C. lectularius* or specific antibodies have not been characterized thus far. We describe a patient with bullous bite reactions after sequential contact with *C. lectularius* over a period of 1 year. In skin tests, we observed immediate reactions to the salivary gland solution of *C. lectularius*, which were followed by a pronounced partially blistering late-phase response. Immunoblot analysis of the patient's serum with salivary gland extracts and recombinant *C. lectularius* saliva proteins revealed specific IgE antibodies against the 32 kDa *C. lectularius* nitrophorin, but not to 37 kDa *C. lectularius* apyrase. Our data demonstrate that bullous cimicosis may be the late-phase response of an allergic IgE-mediated hypersensitivity to *C. lectularius* nitrophorin.

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INTRODUCTION

The common bed bug *(Cimex lectularius)* belongs to the *Cimicidae* family of true bugs. For bites on humans, the species *C. lectularius*, which has a cosmopolitan distribution, is most frequently responsible. Although in the second half of the 20th century *C. lectularius* has been rather scarce in Europe, a dramatic increase of these parasites was noted during the past years, particularly in the UK and the USA (Ter Poorten and Prose, 2005). This increase is thought to be related to the higher incidence of international travel and migration (Paul and Bates, 2000). Common bed bugs are 3–5 mm, oval, flat, hard-bodied, flightless insects that are notorious for their association with humans. Bed bugs may be found in many locations, including homes, hotels, poultry houses, or near bird and bat nests and roosts. Bed bugs often find their way into luggage and may spread in this manner. The generally nocturnal brownish bugs emerge from their hiding places at night for feeding on nearby mammal hosts, including man. Therefore, bed bugs are seen only very rarely and infestation is often just assumed by their offensive odor, which is caused by an oily secretion produced from specialized glands.

The clinical presentation of *C. lectularius* bites substantially varies between individuals, depending on the degree of previous exposure. Typically, the bug bites, lasting 3–15 minutes, are initially not felt, but intensive itching develops after several hours. Variable time periods after the wheal-and-flare response, infiltrated papules, vesicles, or blisters may develop (Sansom *et al.*, 1992; Alexander, 1994). Kemper (1929) demonstrated by self-inflicted bites that upon repeated bug bites, the time interval between bite and reaction decreased from 7 days to 3 hours. Beneath ordinary irritative–toxic reactions, the symptoms may be modulated by the response of the immune system to protein antigens that are present in the saliva (Alexander, 1994). The incidence of immune-mediated allergic hypersensitivity reactions to *C. lectularius* saliva is unknown. Several reports have described bullous reactions to *C. lectularius* in case reports, as well as one local epidemic (Kinnear, 1948; Tharakaram, 1999; Fletcher *et al.*, 2002; Liebold *et al.*, 2003). Moreover, systemic reactions, including asthma or anaphylaxis, to bed bug bites may occur, suggesting an IgE-mediated hypersensitivity (Parsons, 1955; Liebold *et al.*, 2003). However, detailed knowledge of the inflicted antigens of *C. lectularius* saliva responsible for these hypersensitivity reactions is not available to date. Characterization of these antigens may therefore improve and simplify the diagnosis of allergic hypersensitivity

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Abbreviations: PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin

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to *C. lectularius*, and could aid the future development of immunotherapy.

Here, we report on a patient with bullous bite reactions following repeated contact with *C. lectularius* over a period of one year. Upon skin testing, immediate and late-phase reactions were detectable to salivary gland proteins of *C. lectularius*. We then show that the patient's serum contains specific IgE antibodies against the 32 kDa *C. lectularius* salivary gland protein nitrophorin, demonstrating that nitrophorin is an antigen in bullous cimicosis.

RESULTS

Patient

A 43-year-old female patient was referred to the Dermatology Department with a 3-day history of several severely itching erythematous papules and blisters partially arranged in a linear manner on her arms, hands, face, and distal parts of the legs (Figure 1a). She had first noticed several small hemorrhagic pinpoint-sized macules the day before, when leaving a hotel room in a large metropolitan area in England. The patient recalled similar hemorrhagic macules of the extremities a year earlier, when she stayed in a room of the same hotel. At that time, the small hemorrhagic macules healed quickly without therapy in several days, with no development of papules or blisters. At night, the patient had noted small brownish animals in the hotel room during the second visit, which were identified as bed bugs from pictures of several ecto-parasites. Clinical examination revealed multiple erythematous papules, nodules, and blisters at the distal parts of the arms and legs. Several of the isolated nodules revealed an underlying erythema and moderate peripheral whealing up to 10 cm in diameter. Serological analysis did not reveal systemic infection or the presence of autoantibodies. On the right arm, a linear arrangement of erythematous nodules and blisters was noted (Figure 1a). Local treatment with clobetasol propionate 0.5% cream led to a complete remission of the lesions within 10 days.

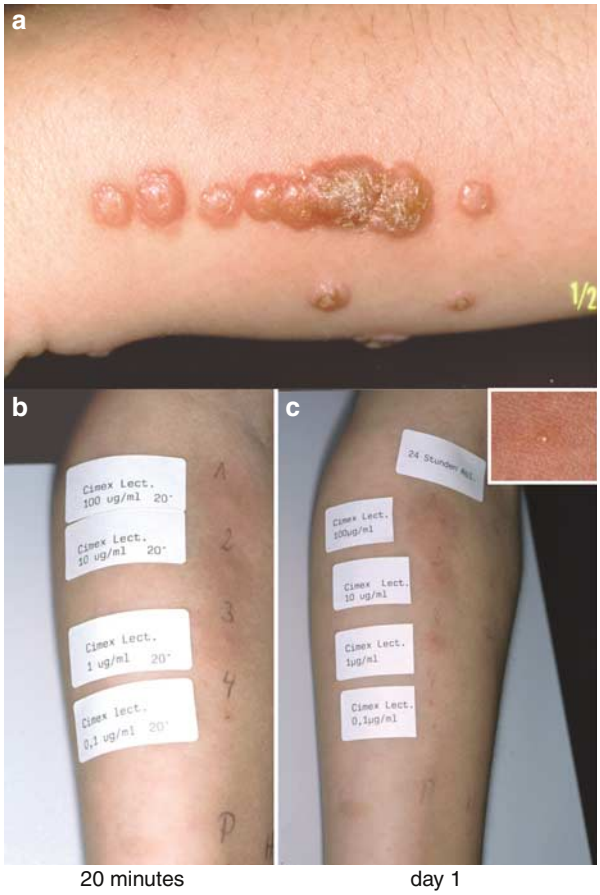

Figure 1. Bullous cimicosis. (**a**) Bite reactions appearing as multiple linearly arranged erythematous nodules with tense blisters on the forearm of the patient. (**b**) SPT with positive immediate reactions to 100 μg/ml (++), 10 μg/ml (++), 1 μg/ml (+), and 0.1 μg/ml (+) after 20 minutes. (**c**) SPT at day 1 revealed infiltrated erythematous papules with a central blister at 100 μg/ml (inset).

Skin test

Immediate-type hypersensitivity to *C. lectularius* was demonstrated by the skin prick test (SPT). Using the *C. lectularius* salivary gland solution at concentrations of 100, 10, 1, and 0.1 μg/ml, we observed wheal and flare responses within 20 minutes, ranging from ++ (100 μg/ml) to + (0.1 μg/ml) (Figure 1b). Papular late-phase reactions peaking at day 1 and decreasing until 3 days after SPT were noted, with a central blister at the highest concentration used (Figure 1c). In contrast, five healthy individuals did not show any positive reaction to the same concentrations of salivary gland solution.

Characterization of the patient's circulating antibodies reveals specific IgE antibodies against *C. lectularius* nitrophorin

Using immunoblot analysis, we next aimed to investigate the presence of specific antibodies to *C. lectularius* salivary gland proteins. Using the salivary gland solution used for skin testing, specific IgE antibodies to a 30–36 kDa protein are detected in the patient's serum, but not in the serum of healthy control individuals (Figure 2a). Total IgE levels in the serum of the patient were marginally increased to 122 kU/l, and IgE levels of the two healthy control individuals used for the immunoblot analysis were 72 and 54, respectively. In contrast, the patient's serum did not contain specific IgG antibodies to *C. lectularius* salivary gland solution even upon intentional overexposure of the blots (Figure 2b). To identify antigenic proteins, we next tested the reactivity of the serum against purified recombinant *C. lectularius* nitrophorin as well as purified recombinant *C. lectularius* apyrase, known abundant *C. lectularius* saliva proteins migrating within this molecular weight range (Valenzuela and Ribeiro, 1998; Valenzuela *et al.*, 1998). We thus expressed and purified *C. lectularius* apyrase and *C. lectularius* nitrophorin and tested the reactivity of our patient's serum against these proteins. IgE immunoblotting revealed IgE antibodies against nitrophorin. Importantly, this reactivity was completely inhibited by preincubation of the serum with 15 μg nitrophorin, but not 15 μg apyrase, in immunoblot inhibition assays using either recombinant nitrophorin (Figure 3b) or *C. lectularius* salivary gland solution (Figure 3c). In contrast,

we could not detect IgE or IgG antibodies to apyrase in our patient's serum (Figure 3a), whereas immunoblotting using rabbit immune serum readily detected apyrase migrating at approximately 48 kDa. Most likely, the higher migration position of recombinant apyrase may represent conformers, non-properly folded, or dimers of the expressed *C. lectularius* apyrase (Valenzuela *et al.*, 1998). In fact, *Cimex* apyrase activity was previously shown to migrate at a larger than predicted molecular weight (Valenzuela *et al.*, 1996, 1998).

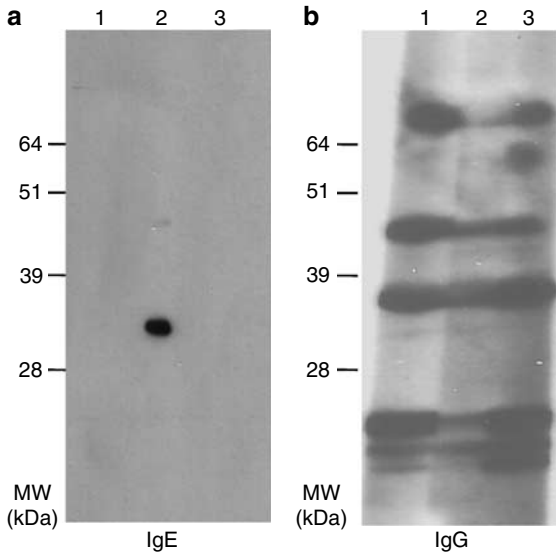

Figure 2. Western blot analysis with *C. lectularius* salivary gland solution. Molecular weight markers are indicated on the left. (**a**) Specific IgE antibodies to a 30–36 kDa protein are detected in the patient's serum (lane 2), but not in healthy control serum (lanes 1, 3). (**b**) Incubation of identical blot strips with the patient's serum (lane 2) compared to healthy controls (lanes 1, 3) did not reveal specific IgG antibodies. An intentional overexposure of the blot strips to possibly detect weak specific antibody binding is shown.

ELISPOT assay

As we had clinically observed late-phase reactions that were paralleled by late-phase reactions in SPT, we also searched for T-cell responses to *C. lectularius* salivary gland solution by ELISPOT assay. However, specific T-cell responses to the *C. lectularius* salivary gland solution were not detectable. Negative control wells containing either peripheral blood mononuclear cells (PBMC) alone or PBMC with buffer alone yielded 0–2 spots per well. The median numbers of PBMC reactive to phytohemagglutinin (PHA) lectine (positive unspecific control) in the patient and in the control person were 527 and 173, respectively.

DISCUSSION

Local sting reactions to insects or parasites are common and a frequent cause for consultation of a dermatologist (Bircher, 2005). Bite reactions in grouped or linear fashion noticed by the patient after waking up in the morning are suspected to be bed bug bites, as in our patient. These late reactions itch intensively and persist over several days. However, widespread bullous reactions to bed bug bites are rare and may obscure the correct diagnosis if adequate patient history is unavailable. Thus, bed bug bites have also to be considered in the differential diagnosis of rapidly evolving bullous eruptions of the skin. Arthropode sting reactions of individuals may represent nonallergic reactions caused by a myriad of only partially known mediators (Bircher, 2005). However, for many arthropodes, as well as bed bugs, it is long known

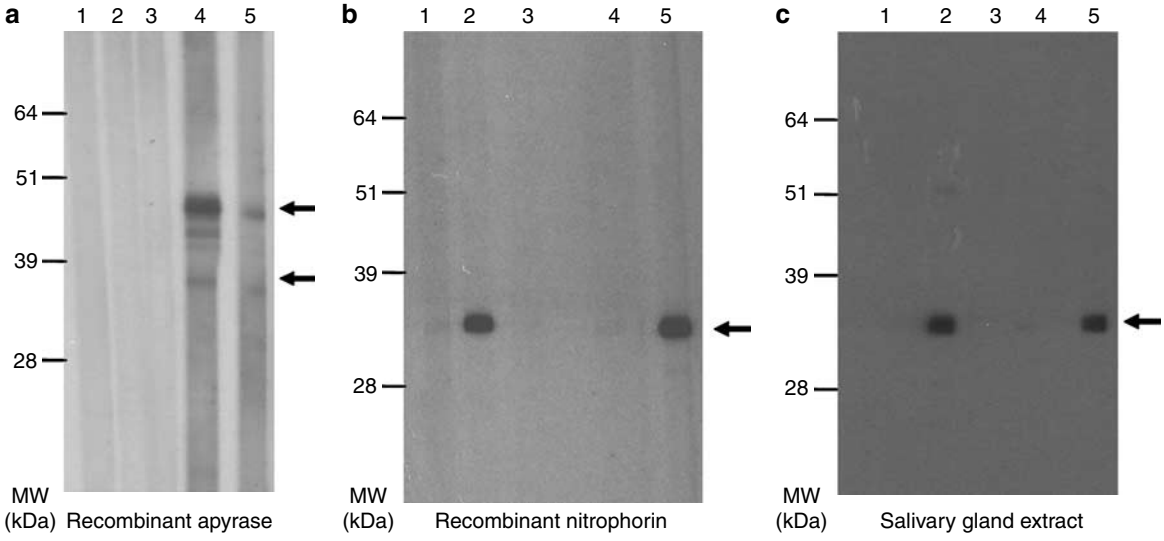

Figure 3. Western blot analysis with recombinant salivary gland proteins from *C. lectularius*. Molecular weight markers are indicated on the left. (**a**) Rabbit antiserum to *Cimex* apyrase (lane 4), but not the serum of the patient (lane 2), preimmune rabbit serum (lane 5), or serum of healthy controls (lanes 1, 3) contain specific IgG antibodies detecting apyrase migrating at approximately 48 kDa. (**b**) Serum of the patient (lane 2), but not of two healthy controls (lanes 1, 3), specifically binds to nitrophorin. Preincubation of the patient's serum with nitrophorin (15 μg; lane 4), but not apyrase (15 μg; lane 5), abolishes the immunoblot reactivity with recombinant *C. lectularius* nitrophorin. (**c**) Immunoblot inhibition using *C. lectularius* salivary gland solution. Serum of the patient (lane 2), but not of two healthy controls (lanes 1, 3), revealed a single strong band with a molecular weight of ~32 kDa, which was completely inhibited by preincubation of the serum with 15 μg of nitrophorin (lane 4), but not apyrase (15 μg; lane 5).

that repeated bites may exhibit immediate wheal and flare symptoms, followed by a papule, sometimes a vesicle or a blister, that suggest immediate- or delayed-type skin reactions to these arthropods (Kemper, 1929; Kinnear, 1948; Mellanby, 1948). These features clearly hint at a hypersensitivity reaction, as also indicated in our case by the increasing symptoms of the patient over a period of 1 year. However, the pathogenesis of the hypersensitivity reaction to *C. lectularius* remains obscure to date. In this report, we have identified a bullous allergic hypersensitivity to bed bug bites mediated by IgE against *C. lectularius* salivary nitrophorin.

We demonstrate in our patient an IgE-mediated type I immune response directed to *C. lectularius* nitrophorin. The skin inflammation after IgE-mediated mast-cell activation occurs as an immediate reaction, starting within minutes, and a late-phase reaction, which takes up to 6–12 hours to develop. Bed bug bites are hardly noticeable, suggesting that our patient most likely suffered the bites during her sleep but did not awaken, noticing papules and subsequent vesicles and blisters by the next morning. The immediate reaction is generated by the activity of mast cell-derived histamine, prostaglandins, and other preformed mediators that mainly cause an increase in vascular permeability (Williams and Galli, 2000). The immediate reaction is followed by the subsequent synthesis and release of leukotrienes, chemokines, and cytokines (Tsicopoulos *et al.*, 1999). In turn, these mediators recruit other leukocytes to the site of inflammation, causing the following late-phase reaction. In bug bites, these late-phase reactions are the main cause for illness due to the development of sustained edema, vesicles, blisters, and intense pruritus (Bircher, 2005).

As an alternative pathophysiological response to bed bug antigens that have diffused into the dermis, a local type III hypersensitivity reaction triggered by circulating specific IgG antibodies is conceivable (Sicherer and Leung, 2005). This Arthus reaction is characterized by locally formed immune complexes binding to receptors on leukocytes and activating complement, which creates a local inflammatory response (Kohl and Gessner, 1999). By immunoblotting, we could not detect circulating specific IgG antibodies to *C. lectularius* salivary gland proteins in the patient's serum. In individuals who have previously been sensitized by repeated bites, a local T-cell-mediated delayed-type hypersensitivity or type IV hypersensitivity reaction may develop over a period of 1–3 days. This response is mediated by antigen-specific T cells, which enter the site of the bite, recognizing complexes of allergen:MHC molecules on antigen-presenting cells and subsequently releasing pro-inflammatory cytokines, such as IFN-γ and tumor necrosis factor-α. These cytokines stimulate the vascular endothelium, enabling the infiltration of additional inflammatory cells. This process usually requires several hours to days, and therefore the fully developed response appears only 1–3 days after the bite (Garcia *et al.*, 2004). However, we were unable to detect specific T-cell responses, suggesting that T-cell activation is either not involved in the pathogenesis or is below the sensitivity level of our ELISPOT assay. As we were able to perform skin testing only 1 year after the patient initially presented to our department due to the slow growth of *C. lectularius* colonies required for the salivary gland preparation, we cannot exclude loss of T-cell reactivity during this period of time. Further studies in additional patients with a hypersensitivity reaction to *C. lectularius* saliva are needed to further clarify this point.

Progress in molecular biology and biochemistry over the past decade has allowed a more detailed understanding of the nature of proteins found in parasite saliva (Valenzuela, 2002). Vertebrates protect themselves against excessive blood loss by activating blood-clotting mechanisms that are induced by platelet aggregation at the site of injury. To feed effectively, blood-sucking arthropods, including *C. lectularius*, have developed various mechanisms that counteract these host responses. *C. lectularius* saliva contains a large array of antiplatelet, anticlotting, and vasodilatory compounds that assist feeding by these animals (Valenzuela and Ribeiro, 1998). During blood sucking, salivary nitric oxide is used as a main vasodilator. The unstable and volatile gas nitric oxide is stored and transported from the salivary glands to the host skin by a 32 kDa heme protein called nitrophorin, which is present abundantly in saliva (Valenzuela and Ribeiro, 1998). Moreover, in order to inactivate ATP released from aggregating platelets, *C. lectularius* saliva contains an apyrase (ATP-diphosphohydrolase) of 37 kDa (Valenzuela *et al.*, 1998). Our study clearly demonstrated an IgE-mediated immune response directed to *C. lectularius* nitrophorin, while no reactivity was found to *C. lectularius* apyrase. Nitrophorin is a protein with a rather limited homology, suggesting that the hypersensitivity of our patient may be restricted to *C. lectularius.* In summary, the bite reactions of our patient are primarily triggered by binding of *C. lectularius* nitrophorin to IgE antibodies on mast cells and a subsequent late-phase response.

Owing to the fact that bed bugs became increasingly uncommon after the Second World War in Europe and the USA, detailed knowledge about potential antigens involved in hypersensitivity reactions is lacking to date. In contrast, the immune reaction to other insects like honey bees or mosquitoes has been studied more intensely, having led to detailed knowledge about antigen nature and B- or T-cell epitopes of specific proteins relevant for the human immune response (Blaser *et al.*, 1998). Ultimately, this knowledge can be used for the future improvement of immune therapeutic strategies for severe systemic reactions (Till *et al.*, 2004). However, an increase of bed bug infestations has been observed over the past decade worldwide, and potential hypersensitivity reactions may pose risks to patients following repeated exposure (Parsons, 1955). Thus, our report provides essential information for future exploration of *C. lectularius* hypersensitivity. Future studies in a larger group of patients with known hypersensitivity to *C. lectularius* will have to clarify whether nitrophorin is the only allergen contained in the saliva of *C. lectularius* once hypersensitivity is suspected in additional patients. To this end, identification, expression, and purification of recombinant allergens provide reagent quantities of protein for future use in serologic testing, to

identify individuals at risk for hypersensitivity to bed bug bites that may ultimately lead to the development of specific immunotherapy for *C. lectularius* antigens for patients at risk.

MATERIALS AND METHODS

Insect rearing and preparation of a *C. lectularius* salivary gland solution

C. lectularius colonies were maintained as described (Valenzuela *et al.*, 1998). Insects were fed every 10 days by exposing them to the shaved abdomen of an anesthetized rabbit. Approximately 100 pairs of salivary glands of insects, 8–10 days after feeding, were dissected and stored in Hepes saline (10 mM Hepes at pH 7.0 in 150 mM NaCl) at −80°C until needed. Before use, salivary glands were thawed and disrupted by sonication. The homogenate was cleared by centrifugation at 14,000 r.p.m. for 5 minutes at 4°C and the resulting *C. lectularius* salivary gland solution was frozen at −80°C until further use.

Skin test

All studies were performed following institutional approval and written informed consent of the patient. The study was conducted according to the Declaration of Helsinki principles. SPT and readings were carried out with serial dilutions of *C. lectularius* salivary gland solution (0.1–100 μg/ml) according to the recommendations of the European Academy of Allergy and Clinical Immunology (1989). A + immediate reaction was defined as a wheal diameter of 2–4 mm, and a ++ reaction as a wheal diameter of 4–6 mm.

ELISPOT assay

Specific T-cell reactivity against salivary proteins of *C. lectularius* was measured by an IFN-γ ELISPOT without prior *in vitro* stimulation. PBMC were collected from the patient and a healthy nonallergic donor as a control person. PBMC were prepared from 20 ml of heparinized blood by density centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway) as described (Leverkus *et al.*, 2003a). HA-Multiscreen plates (Millipore, Burlington, MA) were coated with 100 μl of mouse anti-human IFN-γ antibody (50 μg/ml; clone 1-D1K; Mabtech, Sweden) overnight at 4°C, washed and blocked with human serum for 1 hour at 37°C. PBMC were plated in quadruplicate at 2×10^5/well with 100 μg/ml of *C. lectularius* salivary gland proteins. Negative control wells were included that contained only PBMC, only PBMC with lysis buffer, and medium alone. Positive (nonspecific) control wells containing PBMC with 10 μg/ml PHA lectine (Sigma) were added. After incubation for 20 hours at 37°C, plates were washed with phosphate-buffered saline/0.5% Tween. Bound IFN-γ was detected by adding 100 μl of biotinylated anti-human IFN-γ (2 μg/ml; clone 7-B6-1; Mabtech, Sweden). Spot development was performed using avidin–biotin complexes as described (Herr *et al.*, 1996), and spots were counted using an automated ELISPOT reader system (Carl Zeiss Vision, Germany) with KS ELISPOT 4.5 software.

Expression and purification of recombinant apyrase and nitrophorin

Cimex nitrophorin was expressed as described previously (Weichsel *et al.*, 2005). *Cimex* apyrase full-length cDNA was PCR amplified and cloned into pCRT7NT TOPO expression vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The plasmids were purified and sequenced to confirm the presence and orientation of the *Cimex* apyrase gene. Recombinant *Cimex* apyrase was expressed as a His-tagged protein in *E. coli* strain BL21(DE3)-pLysS (Invitrogen, Carlsbad, CA). The expression culture was sonicated, then centrifuged at $45{,}500 \times g$ for 40 minutes and the pellet was suspended in 50 mM Tris, pH 8.0, 20 mM EDTA (TE 50/20), and complete protease inhibitor mix (Roche Laboratories, Indianapolis, IN). The sample was centrifuged at $25{,}000 \times g$ for 2 hours and the pellet was washed several times over 2 days in TE 50/20 with complete protease inhibitor mix. The pellet was solubilized in inclusion body solubilization reagent (Pierce, Rockford, IL) and dialyzed against 50 mM Tris, pH 8.0, overnight. The sample was washed with TE 50/20 as mentioned and the pellet was solubilized in 7 M guanidine hydrochloride. The His-tagged protein was purified using MagneHis protein purification system (Promega, Madison, WI) and dialyzed against TE 50/20. To confirm the identity and purity of the recombinant protein, the sample was run in SDS-PAGE 4–12% and sequenced by Edman degradation. The rest of the sample was lyophilized until further use.

Immunoblot analysis

A salivary gland solution of *C. lectularius* or purified recombinant *C. lectularius* apyrase or nitrophorin was separated by SDS-PAGE as described previously, with minor modification (Leverkus *et al.*, 2003b; Wachter *et al.*, 2004). Briefly, 1–2 μg of protein was heated for 7 minutes at 70°C in reducing sample buffer and separated by 4–12% gradient gel SDS-PAGE (Invitrogen, Karlsruhe, Germany). The proteins were then electrotransferred onto nitrocellulose membranes. Membranes were blocked by incubation with 5% (wt/vol) milk powder dissolved in 0.2 mol/l phosphate-buffered saline supplemented with 0.5% (vol/vol) Tween (AQTPBS) for 2 hours at room temperature. The immunoblot was completed by incubation of the membranes with patients' sera (1:10 dilution) at 4°C overnight. After washing three times with TPBS, membranes were incubated with the appropriate dilutions of monoclonal anti-human IgE (1:750; Binding site, Heidelberg, Germany) or anti-human IgG antibodies (1:500; Jackson Immunoresearch Laboratories, West Grove, PA) for 60 minutes at room temperature. Following three washes with TPBS, bands were visualized using the ECL detection kit (Amersham).

Immunoblot inhibition assay

Immunoblot inhibition assays were performed using purified recombinant apyrase and nitrophorin as described recently (Schad *et al.*, 2005). Briefly, the serum of the patient was preincubated for 60 minutes with either 15 μg of apyrase or nitrophorin, and subsequently added to blot strips carrying purified recombinant nitrophorin or separated salivary gland solution, respectively. The next steps were performed as described above.

CONFLICT OF INTEREST

The author states no conflict of interest.

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