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Erysipelas Caused by Group A Streptococcus Activates the Contact System and Induces the Release of Heparin-Binding Protein

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Bacterial skin infections, such as erysipelas or cellulitis, are characterized by fever and a painful erythematous rash. Despite the high prevalence of these infections, little is known about the underlying pathogenic mechanisms. This is partly due to the fact that a bacterial diagnosis is often difficult to attain. To gain insight into the pathogenesis of erysipelas, we investigated the samples obtained from infected and noninfected areas of skin from 12 patients with erysipelas. Bacterial cultures, detection of specific streptococcal antibodies in convalescent sera, and immunohistochemical analyses of biopsies indicated group A streptococcal etiology in 11 of the 12 patients. Also, electron micrographs of erythematous skin confirmed the presence of group A streptococcal cells and showed a limited solubilization of the surface-attached M protein. Degradation of high-molecular-weight kininogen and upregulation of the bradykinin-1 receptor in inflamed tissues indicated activation of the contact system in 11 patients. Analyses of release of the vasoactive heparin-binding protein (HBP) showed increased levels in the infected as compared with the noninfected areas. The results suggest that group A streptococci induce contact activation and HBP release during skin infection, which likely contribute to the symptoms seen in erysipelas: fever, pain, erythema, and edema.

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INTRODUCTION

Erysipelas is a rapidly spreading infection of the superficial layers of the skin often accompanied by systemic symptoms such as fever, chills, and malaise. The progressing erythema is characterized by a clear distinction from surrounding tissue. Bacteriological diagnosis of the disease is often difficult. Blood cultures are positive in <5% of cases (Perl *et al.*, 1999) and culturing of needle aspirations or punch biopsies from skin lesions has limited value (Newell and Norden, 1988; Duvanel *et al.*, 1989; Lebre *et al.*, 1996). Cultures together with other available evidence such as serological data indicate that the group A streptococcus is the most prevalent pathogen, but other β-hemolytic streptococci have also been implicated (Eriksson *et al.*, 1996; Rantala *et al.*, 2009).

The human contact system (Bhoola *et al.*, 1992; Colman and Schmaier, 1997; Joseph and Kaplan, 2005) consists of three serine proteinases: coagulation factor XI and XII, plasma kallikrein, and the nonenzymatic cofactor high-molecular-weight kininogen (HK). Activation of the contact system has three potential consequences: the initiation of the intrinsic pathway of coagulation, the generation of antibacterial peptides, and the release of bradykinin, a proinflammatory peptide that increases vascular permeability and induces fever and pain. Bradykinin is generated when HK is cleaved by activated plasma kallikrein into different protein fragments.

Previous studies have shown that several bacterial species, such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella*, and group A streptococci, can assemble components of the contact system at their surface, resulting in bradykinin release (Ben Nasr *et al.*, 1996; Herwald *et al.*, 1998; Mattsson *et al.*, 2001). In group A streptococci, HK is bound to the bacteria by the cell-wall-attached M protein. When HK is associated to the bacterial cell surface, it is exposed to a secreted streptococcal cysteine proteinase (SpeB) that cleaves HK and releases bradykinin both *in vitro* and in animal models of infection (Herwald *et al.*, 1996).

The M protein of group A streptococci has recently been shown to interact with neutrophils by a previously unidentified mechanism. M proteins released from the streptococcal cell wall form complexes with fibrinogen, which activate

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Abbreviations: HBP, heparin-binding protein; HK, high-molecular-weight kininogen; PVDF, polyvinylidene difluoride

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neutrophils by binding to β2 integrins (Herwald *et al.*, 2004). This leads to the secretion of the heparin-binding protein (HBP), which is an inflammatory mediator that exert its effect on the capillary endothelial cell layer and induces vascular leakage (Gautam *et al.*, 2001). It was shown that M protein, when injected into mice, could induce severe pulmonary damage characterized by leakage of plasma and blood cells (Herwald *et al.*, 2004; Soehnlein *et al.*, 2008).

The skin lesion seen in patients with erysipelas has all the characteristics of inflammation: an intense and painful erythema with edematous elevation of the affected skin, signs that could be explained, at least in part, by the action of bradykinin and HBP released in response to group A streptococcal infection. The aim of this investigation was therefore to analyze whether the contact system and neutrophils are activated in patients with erysipelas, and thus help explain the pronounced local inflammation that is characteristic of this disease.

RESULTS

Patient characteristics, bacterial cultures, and serologic testing

The 12 patients included in the study had typical signs of erysipelas with fever and a characteristic erythema on one of the legs. They were all admitted to hospital and treated with antibiotics, and showed signs of recovery with absence of fever and a less intense erythema within 3 days. A positive blood culture for group A streptococci was found in one of the patients (patient 11). Cultures were also taken from the wound in four patients who had superficial skin wounds present at the same leg as the erysipelas lesion. Two of these patients were positive for group A streptococci, one for *S. aureus*, and one patient had a mixed culture of both *S. aureus* and group G streptococci (Table 1). Bacteriological diagnosis by serologic testing was also carried out. IgG antibody levels against seven different group A streptococcal antigens, previously reported to evoke antibody responses during group A streptococcal infections (Åkesson *et al.*, 2004), were determined in acute and convalescent serum samples. Seven of the twelve patients developed an increased antibody titer, defined as a >50% increase of the ELISA index, in the convalescence serum against at least two of the streptococcal antigens (Table 1).

Table 1. Patient demographics and laboratory data of patients with erysipelas

Patient	Sex	Age (years)	Prior illness	Onset days (*n*)	Antibiotic doses (*n*)	Bacteriologic culture	Serology (GAS)
1	M	62	Diabetes	3	3	—	–
2	F	50	Healthy	2	3	*S. aureus*	–
3	F	77	Healthy	2	4	—	+
4	M	61	Healthy	2	2	—	+
5	M	63	Diabetes	1	2	—	–
6	M	29	Healthy	2	3	GAS	+
7	F	55	Healthy	3	3	—	+
8	F	68	Diabetes	1	2	—	–
9	F	88	Healthy	3	2	*S. aureus* + GGS	–
10	M	50	Healthy	1	2	—	+
11	F	85	Healthy	2	3	GAS	+
12	F	58	Healthy	2	3	—	+
Mean		62		2	2.5		
Median		61.5		2	3		
Range		29–88		1–3	2–4		

Abbreviations: F, female; GAS, group A streptococci; GGS, group G streptococci; M, male.
Onset days, number of days of disease prior to biopsy; antibiotic doses, number of antibiotic doses before the time of biopsy; bacteriological culture, bacteriological findings from blood and wound cultures; serology, serology results from analysis of acute and convalescent sera against seven different GAS antigens.

HK is degraded and HBP is released in the infected skin tissue

Activation of the contact system results in a proteolytic cleavage of HK and the release of bradykinin. Bradykinin is a small peptide that is cleared rapidly from tissue, and the analysis of HK degradation is therefore a more reliable measure of activation. Homogenized skin biopsies from infected and noninfected areas were analyzed using western blot. Antibodies against HK showed one 120 kDa band of the same size as native HK in all samples, and one band of 63 kDa, which could correspond to either low molecular weight kininogen or the heavy chain of HK (Figure 1). In the material obtained from infected skin, additional immunoreactive bands were observed. Their molecular masses of 58 and 45 kDa correlate well to products observed after cleavage of HK (Kahn *et al.*, 2002), showing a degradation of the protein (Figure 1a). In the controls taken from the healthy tissue of the other leg, HK was degraded in only one of the patient samples (Figure 1b).

The presence of HBP in the tissue samples was also investigated. In another immunoblot of proteins extracted from the biopsy specimens, antibodies against HBP detected a band of the expected size of 29 kDa (Figure 2). HBP was present in the skin tissue obtained from the infected area of 11 of the 12 patients, but only in one of the samples taken from the healthy legs.

Inflammatory responses to group A streptococci in tissue sections

Group A streptococci is regarded as the principal etiological agent in erysipelas, but only seven patients could be diagnosed with a group A streptococcal infection by culturing or serologic testing. Tissue sections were therefore analyzed by immunohistochemical staining using antibodies to specific carbohydrate components of the group A streptococcal cell wall. Also, sections were stained with antibodies against the streptococcal SpeB, which is secreted from all serotypes of group A streptococci. This assay revealed the presence of group A streptococci in the tissue of 11 of the 12 patients (Table 2). The only patient whose tissue was

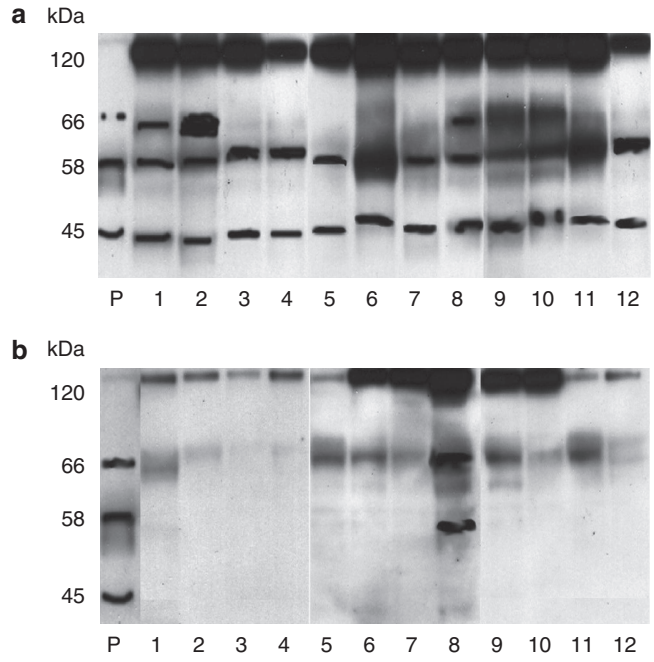

Figure 1. Proteolytic cleavage of HK in skin biopsies. Proteins were extracted from skin biopsies from the leg with signs of inflammation (**a**) or of the other noninfected leg (**b**) and subjected to SDS–PAGE. The gels were transferred to a polyvinylidene difluoride membrane and probed with polyclonal antibodies raised against high-molecular-weight kininogen (HK). Patients are numbered 1–12. Lanes marked ''P'' contain human plasma stimulated with dextran sulfate, leading to degradation of intact HK.

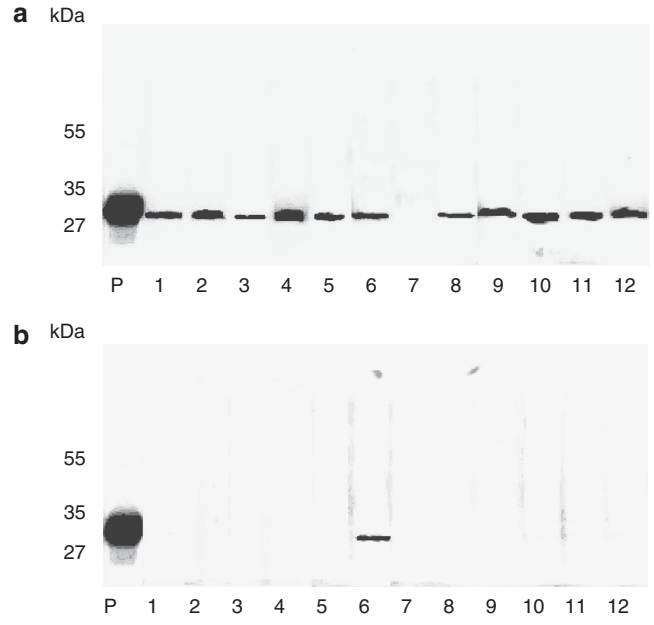

Figure 2. Presence of heparin-binding protein (HBP) in skin biopsies. Extracted proteins from tissue taken from infected skin (**a**) or the contralateral healthy-looking leg (**b**) of patients 1–12 were analyzed by immunoblotting using a polyclonal antiserum against HBP. Lanes marked ''H'' contain purified recombinant HBP.

negative for both group A streptococci and SpeB was culture positive for *S. aureus*.

To further investigate the inflammatory responses triggered by contact activation and the granular shedding of neutrophils, we stained the sections for the bradykinin-1 receptor (BK-R1) and HBP. The expression of BK-R1 is induced by IL-1β but also by bradykinin and its metabolites, and thus an indicator of contact activation. Both BK-R1 and HBP were present in all skin sections, however in varying amounts when analyzed semiquantitatively (Table 2). A more detailed analysis was performed in one of the patients (patient 11), including analyses of two additional biopsies. One biopsy was taken approximately 5 cm outside the margin of the erythema and one biopsy from a corresponding region from the other, noninfected leg. Staining intensities were higher for all the studied antigens in the inflamed skin as compared with the tissue adjacent to the infection or from the opposite leg (Figure 3a). The biopsies were also stained for neutrophils, which revealed a heavier infiltration of neutrophils in the epicenter biopsy than in other sites (data not shown). Also, a quantitation of antigen expression by acquired computerized image analysis (ACIA) confirmed a considerably higher prevalence of antigens in the erythematous area. However, bacterial cell wall antigens and HBP were also detected in tissue outside the apparent infection (Figure 3b). In addition, analysis by confocal microscopy showed that group A streptococcus were present in the epicenter of the erysipelas infection (Figure 3c).

M protein is cleaved from the group A streptococcal surface during skin infection

To visualize infecting bacteria at higher magnification, we also analyzed tissue sections from three of the patients (patients 1, 4, and 6) using transmission electron microscopy. Figure 4a shows spherical cells in the dermis of the erythematous skin of all patients. The structures are suggestive of bacteria with a diameter of 0.6–1 μm. An experiment with tissue sections from the same patients incubated with gold-labeled antibodies against streptococcal cell wall antigens, i.e., the group A carbohydrate, indicated that the cells were indeed group A streptococci (Figure 4b). Compared with previous electron micrographs of group A streptococci grown in culture media (Fischetti, 1989; Herwald *et al.*, 2004), the bacteria identified in the erythematous skin seemed to lack the hairlike structures representing the surface-attached M proteins. Further analyses showed that antibodies directed toward the cell-wall-associated and -conserved C-terminal part of the M1 protein bound to the bacteria (Figure 4c). The protruding N-terminal part of the M proteins is highly variable among group A streptococci of different serotypes. However, a common property of these heterogeneous N-terminal domains is the ability to bind fibrinogen. When skin sections were incubated with gold-labeled human fibrinogen, no colocalization was observed with bacterial surfaces (Figure 4d). The results suggest that the N-terminal portion of the M protein is cleaved off when group A streptococci infect the skin.

Table 2. Immunohistochemistry data of samples from patients with erysipelas

Patient	Site of biopsy	GAS	SpeB	BK-R1	HBP
1	Epicenter	++	++	+	+
2	Epicenter	0	0	+	+
3	Epicenter	+	+	+	+
4	Epicenter	++	++	++	++
5	Epicenter	(+)	++	+	+
6	Epicenter	++	++	+	+
7	Epicenter	++	++	++	++
8	Epicenter	++	++	++	++
9	Epicenter	+	++	+	+
10	Epicenter	+	+	+	+
11	Epicenter	++	++	++	++
	5 cm distal	+	(+)	+	++
	Healthy leg	(+)	(+)	(+)	+
12	Epicenter	++	++	+	++
	Healthy leg	+	+	+	0

Abbreviations: BK-R1, bradykinin receptor 1; GAS, group A streptococcus; SpeB, streptococcal pyrogenic exotoxin B; HBP, heparin-binding protein; 0, completely negative; (+), faint staining; +, few positive cells or low expression of antigen; ++, high frequency of positive cells or antigen.

DISCUSSION

During acute inflammation, plasma components, and leukocytes are transported to the infected tissue and interact to defend against bacterial colonization, multiplication, and dissemination. In this study, two different inflammatory mechanisms during group A streptococcal infection were studied: the triggering of the contact system and the release of HBP from neutrophils.

Extensive proteolysis of HK was observed in all tissue specimen obtained from the tender and erythematous skin of the erysipelas infection. Also, the resulting HK fragments were of the same molecular weight as shown in a previous study showing contact activation in plasma during vasculitis (Kahn *et al.*, 2002). A heterogeneous degradation of the 65 kDa heavy chain of H kininogen was detected among the patients. A possible explanation is varying activities in the samples of other proteolytic enzymes known to degrade H kininogen. For example, granulocyte elastase has been shown to cleave H kininogen without destroying its bradykinin portion (Kleniewski and Donaldson, 1988). The local degradation of HK in infected skin is only an indirect sign of bradykinin release, but the upregulation of BK-R1 in the tissue sections strongly suggests the presence of bradykinin. It is known that HK can be activated on the surface of endothelial cells (Colman and Schmaier, 1997), and that bradykinin can be generated at the luminal side of the endothelium (Reddigari *et al.*, 1995). It has previously been

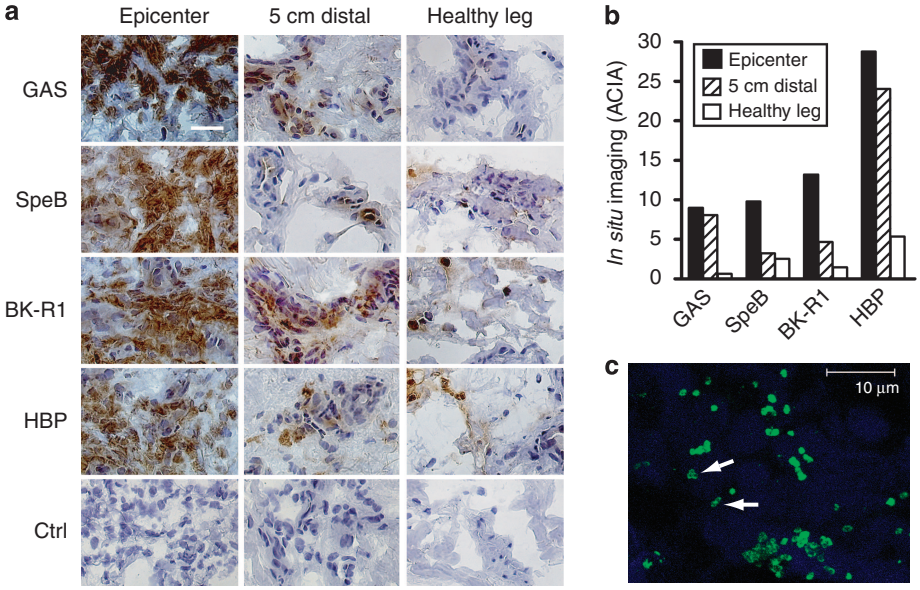

Figure 3. Host–pathogen responses at the local tissue site of infection. Tissue biopsies from the inflamed leg were obtained from the epicenter, 5 cm outside the lesion, and from the healthy leg of patient 11. Biopsies were sectioned, stained for group A streptococci (GAS), streptococcal pyrogenic exotoxin B (SpeB), bradykinin-1 receptor (BK-R1), heparin-binding protein (HBP), and analyzed by *in situ* imaging as described in Material and Methods. A control staining where the primary antibody was omitted was also performed (ctrl). (**a**) Representative immunohistochemically stained areas, (**b**) acquired computerized image analysis (ACIA) values of the whole tissue section. Group A streptococci were also identified by immunofluorescence staining and confocal microscopy analyses (**c**). Note the distinct positively stained cocci indicated by arrows. Scale bars = 30 µm (**a**) and 10 µm (**c**).

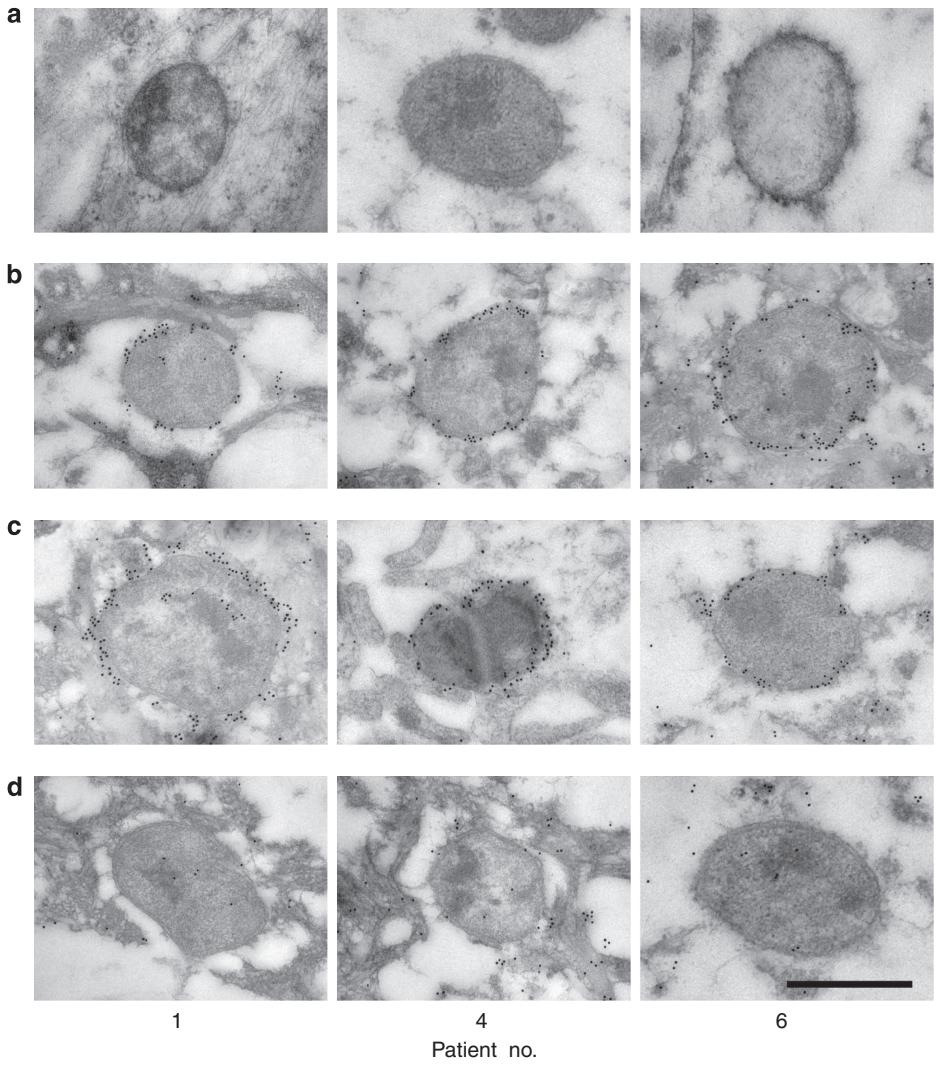

Figure 4. Transmission electron microscopy of thin sections taken from the skin of three patients with erysipelas. (**a**) Spherical bacteria-like structures are occasionally seen in the dermis. Sections were incubated with a specific antiserum against the Lancefield group A carbohydrate (**b**) or an antiserum raised against the conserved C-terminal region of the M1 protein (**c**), followed by detection with gold-labeled secondary antibodies. Colocalization of group-A-specific carbohydrate and M protein with bacterial cell surfaces is visible. (**d**) Sections were also incubated with gold-labeled fibrinogen. Scale bar = 0.5 μm.

shown that plasma bradykinin levels are elevated in patients with sepsis caused by *S. aureus*, and it has been speculated that uncontrolled contact activation contributes to the nonbeneficial inflammatory response seen in sepsis (Sriskandan and Cohen, 2000; Mattsson *et al.*, 2001). In this study, contact activation was detected in the nonaffected leg in only one patient, and in the patient sampled from an area just outside the margin of erythema, the expression of BK-R1 was markedly lower. This reflects a response that is limited and localized to the area of bacterial invasion. The inflammatory properties of bradykinin could be advantageous to the host by causing pain and an influx of plasma component to the tissue by increasing vascular permeability. Conversely, several bacterial species secrete proteolytic enzymes that cleave HK and generate bradykinin (Imamura *et al.*, 2004), suggesting that the physiological effects of bradykinin is favorable for the bacterium under certain conditions.

Wound cultures and analyses of convalescent sera revealed a likely group A streptococcal etiology in seven out of the twelve patients. When tissues were analyzed using immunohistochemistry and confocal microscopy, however, group A streptococci were detected in all patients but patient 2. This underlines the difficulties in obtaining a bacterial diagnosis of clinical skin infections, but shows that 11 of 12 patients in this study were infected with group A streptococci. Also, the data suggest that bacterial cells are present throughout the inflamed skin area. This supports the notion that inflammation is induced close to the bacterial surfaces, and not only as a distant effect of streptococcal toxins.

Transmission electron microscopy also detected group A streptococcal cells in the epidermis and dermis of the infected skin. Antibody analyses of the sections showed that the surface-attached M proteins of the bacteria had been processed. M protein binds fibrinogen with high affinity

(Kantor, 1965), and this interaction has been mapped to the variable N-terminal region of the M protein extending from the bacterial surface (Åkesson *et al.*, 1994). It was previously shown that when group A streptococci were incubated with SpeB, fibrinogen-binding fragments of M protein were solubilized (Berge and Björck, 1995). The lack of colocalization of fibrinogen to bacterial surfaces in the electron micrographs suggests that biologically active M protein fragments are released also during superficial skin infection. As mentioned, circulating complexes of M protein and fibrinogen are able to release neutrophil-derived HBP (Herwald *et al.*, 2004). The vascular leakage caused by elevated levels of HBP is thought to be important in severe streptococcal infections by inducing disturbed microcirculation, hypotension, and organ failure. HBP has previously been detected in tissue samples taken from patients with group A streptococcal necrotizing fasciitis (Herwald *et al.*, 2004; Påhlman *et al.*, 2006). The local presence of HBP in patients with erysipelas suggests the same mechanism also during this milder condition. Severity of disease could thus be related to differences in bacterial load, M protein expression, or proteolytic activity.

A number of preclinical investigations have been performed on the interactions between group A streptococci and the contact system and neutrophils. This investigation has been an attempt to study some of these mechanisms in a clinical setting including patients with superficial skin infections. The partial cleavage of the M protein from the bacterial surface, the extensive degradation of HK and upregulation of BK-R1, suggesting increased bradykinin levels, and the presence of HBP in infected tissue, all fit into previously suggested pathophysiological models. Finally, the study confirms that the group A streptococcus is the predominant etiological agent in spreading superficial infections of the skin.

MATERIALS AND METHODS

Patients, skin biopsies, and serum samples

Twelve patients presenting with erysipelas were enrolled in a prospective nonconsecutive study at the Clinic for Infectious Diseases, Lund University Hospital, Lund, Sweden, during 2004–2006. Skin biopsies were obtained from the patients after informed written consent, according to protocols approved by the ethics committee at Lund University and in accordance with the Declaration of Helsinki Principles. The inclusion criteria were a typical erythema localized on one of the lower extremities, and fever $>38\,^{\circ}C$. No patients with a history of recurrent erysipelas, malignancies, radiation therapy, immunodeficiency, or immunosuppressive therapy were included. The median age of the patients was 61.5 years (range 29–88 years). The median time from the onset of disease (erythema or fever) to inclusion was 2 days (range 1–3 days), and all the patients had received antibiotic therapy before inclusion (Table 1).

At the time of inclusion, two punch biopsy specimens (4 mm) were obtained from the center of the lesion of each patient. One of the biopsies was snap-frozen and stored at $-80\,^{\circ}C$ and later analyzed using protein extraction and western blot. The other specimen was longitudinally cut in half and prepared for immunohistochemistry and electron microscopy. From each patient an additional skin biopsy specimen was taken from the corresponding area of the other noninfected leg, and from two of the patients (patients 11 and 12) a third biopsy was taken from the affected leg at a distance of 5 cm outside the advancing margin of the erythema. These samples were stored frozen at $-80\,^{\circ}C$. Acute serum samples of all patients were obtained at inclusion. Blood cultures and cultures from skin wounds, when present at the same leg as the erythematous lesion, were obtained before antibiotic therapy was started. Each patient had a follow-up visit after 3–5 days and a final control after 27–75 days, when a convalescence serum sample was collected. No recurrences were noted during this follow-up period.

ELISA

Antibody levels in patient sera against the extracellular group A streptococcal proteins SpeB, SclB, MtsA, IdeS, and EndoS were measured using an ELISA assay. The bacterial antigens and the details of the assay were described previously (Åkesson *et al.*, 2004). An antigen concentration of $0.5\,mg\,ml^{-1}$ was used for coating microtiter plates (Maxisorp; Nunc, Rochester, NY). Antibodies toward streptococcal DNAse and streptolysin were analyzed by standard methods (enzyme inhibition test and latex agglutination test, respectively) at the Department of Clinical Immunology, Lund University Hospital.

Western blot

Skin samples of approximately $2 \times 2 \times 4$ mm were homogenized by mechanical disruption in 0.4 ml 10% SDS. After incubation at $99\,^{\circ}C$ for 20 minutes, followed by centrifugation for 5 minutes at 3,000 r.p.m., the supernatants were collected. The protein content in each supernatant was measured by optical density (Advanced Protein Assay Reagent; Cytoskeleton, Denver, CO) and adjusted by dilution in 10% SDS so that the total protein concentrations were equal in all the samples. SDS sample buffer (10 µl) was added to supernatant (10 µl) and incubated at $95\,^{\circ}C$ for 5 minutes. Proteins were separated on a 4–12% NuPAGE Novex Bis-Tris gel (Invitrogen, Carlsbad, CA), and transferred from the gel onto a polyvinylidene difluoride membrane (Immobilon P, Millipore, Beillerca, MA) by electrophoretic transfer according to the manufacturer's instructions. The membranes were blocked in phosphate-buffered saline with 5% dry milk powder and 0.05% Tween 20. Presence of kininogens was determined with antiserum AS88 (1:5,000 dilution; Müller-Esterl *et al.*, 1988). The second antibody was an anti-sheep IgG (1:3,000) conjugated with horseradish peroxide (ICN Biomedicals, Costa Mesa, CA). HBP was detected by a polyclonal anti-HBP rabbit antiserum (1:5,000 dilution; Lindmark *et al.*, 1999), and a horseradish-peroxide-labeled anti-rabbit secondary antibody (Bio-Rad, Hercules, CA). Immunoreactive protein bands were visualized with an electrochemiluminescence kit (Pierce, Piscataway, NJ). As a positive control for HK proteolysis, dextran sulfate (Sigma, St Louis, MO) was added to human plasma. Plasma was incubated with dextran sulfate in the presence of 2 mM zinc at a final concentration of 75 µM at $37\,^{\circ}C$ for 1 hour. Recombinant HBP used as a control was produced and purified as previously described (Rasmussen *et al.*, 1996).

Immunohistochemical staining and confocal microscopy

Skin biopsies from patients were cryosectioned, fixed, stained, and analyzed using image analysis as previously described (Norrby-Teglund *et al.*, 2001). Group A streptococci were identified

using a polyclonal rabbit antiserum specific for the Lancefield group A carbohydrate (Difco, Detroit, MI), and the *in vivo* expression of SpeB was determined by a polyclonal rabbit antisera raised against native SpeB (Toxin Technology, Sarasota, FL). HBP and BK-R1 were identified by purified IgG from respective polyclonal anti-HBP and anti-BK-R1 rabbit antiserum (Lindmark *et al.*, 1999; Lamb *et al.*, 2002). The secondary antibody was a biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA).

Immunohistochemical stainings were analyzed by ACIA. The whole tissue section was included in the analysis, which yielded an analyzed cell area (defined by the hematoxylin counterstaining) ranging from 0.6×10^5 to 1.3×10^6 μm^2. The results are presented as ACIA value, which equals percent positively stained area $\times$ mean intensity of positive staining.

Immunofluorescence staining of group A streptococci was performed and analyzed using confocal microscopy. The staining procedure has previously been described in detail (Thulin *et al.*, 2006). Streptococci were identified using a specific rabbit antiserum against the Lancefield group A carbohydrate. For evaluation, the Leica (Wetzlar, Germany) confocal scanner TCS2 AOBS with an inverted Leica DMIRE2 microscope was used.

Electron microscopy

Thin sections were subjected to immunolabeling as previously described (Roth, 1986), except that Aurion-BSA (Aurion, Wageningen, the Netherlands) was used as a blocking agent. Briefly, the sections were placed on grids and incubated with a specific antiserum against the Lancefield group A carbohydrate or a rabbit antiserum directed against the conserved C-terminal part of the M1 protein (Åkesson *et al.*, 1994), followed by immunodetection with 10 nm gold beads on a secondary antibody against rabbit IgG (Agar Scientific, Stansted, UK). Alternatively, specimens were incubated with human fibrinogen (Kabi) coupled to colloidal gold before the final washing and fixation steps. After further washes in incubation buffer, the sections were postfixed in 2% glutaraldehyde. Finally, sections were washed in distilled water, poststained with uranyl acetate and lead citrate, and observed in a Jeol (Tokyo, Japan) JEM 1230 transmission electron microscope, operated at an accelerating voltage of 80 kV. Images were recorded with a Gatan (Pleasanton, CA) MultiScan 791 CCD camera.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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