Correlation between serum reactivity to Demodex-associated Bacillus oleronius proteins, and altered sebum levels and Demodex populations in erythematotelangiectatic rosacea patients

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Rosacea is a chronic inflammatory condition that affects the skin of the face and the eyes. The aetiology of rosacea is not clearly established but increasing evidence suggests a potential role for bacteria in the induction of the condition. A role for Bacillus oleronius, originally isolated from within a Demodex folliculorum mite, in the aetiology of the condition has been suggested. The aim of the study was to determine whether a correlation existed between the level of sebum and the density of D. folliculorum on the face of Bacillus positive rosacea patients, and the reactivity of these patients’ sera to proteins of B. oleronius. Serum reactivity to the 62 and 83 kDa B. oleronius proteins was found in 82.6 % (62/75) of the rosacea patients and in 26.9 % (14/52) of controls (P<0.0016). In the group of rosacea patients whose sera reacted to B. oleronius proteins, the level of sebum was statistically lower than in controls (P<0.01). The density of D. folliculorum on the face of Bacillus positive rosacea patients was statistically higher than controls (P<0.0001). Rosacea patients demonstrated increased Demodex populations on their faces and reduced sebum levels. Their sera also showed reactivity to B. oleronius proteins, suggesting a potential role for this bacterium in the aetiology of rosacea.

INTRODUCTION

Rosacea is a common, chronic, multiphase inflammatory dermatosis of the face, the course of which is characterized by periods of exacerbation and remission (Wilkin et al., 2002). The most frequent skin changes in rosacea patients include flushing or permanent erythema, papules, pustules and telangiectasias, located in the central part of the face i.e. cheeks, nose, chin and forehead (Wilkin et al., 2002; Crawford et al., 2004). Four basic subtypes of rosacea have been identified: erythematotelangiectatic, papulopustular, phymatous and ocular (Wilkin et al., 2002). Erythematotelangiectatic rosacea is characterized by extensive erythema and oedema on facial skin (Wilkin et al., 2002) and may be controlled by the use of selected antibiotics or by pulsed dye laser (PDL) therapy (Gupta & Chaudhry, 2005). However, the inflammation associated with papulopustular rosacea tends to be centred on the pilosebaceous unit (Jarmuda et al., 2012; Holmes, 2013).

Various factors such as vascular and immunological abnormalities, agents responsible for degradation of the structures of connective tissue and selected infectious causes are believed to play a role in the aetiology of rosacea (Gupta & Chaudhry, 2005; Yamasaki & Gallo, 2009). Since the aetiology of the disease remains unclear, the treatment of rosacea presents a challenge to the clinician and requires a highly individual approach. Management with antibiotics, mostly from the group of tetracyclines, macrolides and metronidazole, is generally recommended (Pelle et al., 2004).

The possible role of Demodex folliculorum mites in the pathogenesis of rosacea, especially the mechanism of passive transfer of other micro-organisms, has been speculated upon for many years (Jarmuda et al., 2012). The incidence of Demodex on the facial skin of patients with rosacea is significantly higher than in controls (Bonnar et al., 1993). A significantly greater density of the mites per cm² was detected in patients with papulopustular rosacea (PPR) (Bonnar et al., 1993) and the composition of the lipids from
their sebum revealed differences in comparison with controls, which might facilitate the development of larger populations of mites (Ni Raghallaigh et al., 2012). The presence of *D. folliculorum* in the sebum secretions from the pilosebaceous unit was found in 90.2 % of PPR patients and only in 11.9 % of healthy controls. Additionally, histological tests of skin samples obtained from these patients revealed that the presence of *Demodex* was strongly correlated with substantial perifollicular lymphocytic infiltration (Georgala et al., 2001). *Bacillus oleronius* was successfully isolated from a *Demodex* mite obtained from a PPR patient (Lacey et al., 2007), where it may play a role in facilitating digestion, as it does in the termite (Kühnigk et al., 1995). This bacterium produced two highly immunogenic proteins that showed reactivity to sera from PPR (Lacey et al., 2007), ocular (Li et al., 2010) and erythematotelangiectatic (O’Reilly et al., 2012c) rosacea patients. It has been suggested that the release of *B. oleronius* proteins from dead *Demodex* mites may lead to neutrophil recruitment and activation in the vicinity of the pilosebaceous unit (O’Reilly et al., 2012a), thus possibly explaining why the inflammation in rosacea is often centred around this structure. The potential role of these bacterial proteins in inducing corneal damage in ocular rosacea has been described (O’Reilly et al., 2012b).

The aim of the work presented here was to establish whether a correlation existed between the sebaceous condition of the skin, the density of *Demodex* mites and reactivity of sera obtained from rosacea patients to *B. oleronius* proteins, in order to determine the role of *B. oleronius* in the induction of this disfiguring condition.

**METHODS**

**Study population.** Seventy-five patients with erythematotelangiectatic rosacea (33 males and 42 females), Fitzpatrick skin phototypes I or II, aged 20–81 years, hospitalized between 1 February 2011 and 16 December 2011 at the Dermatology Clinic, Poznań University of Medical Sciences or treated at the out-patient Dermatology Clinic, were enrolled in the study. Mean age of rosacea patients was 47.07 years (females, 44.95; males, 49.76). Patients did not receive any oral antibiotics, retinoids, glucocorticosteroids or sulfones for at least 3 months prior to recruitment to the study.

Fifty-two volunteers (28 females and 24 males), aged 18–89 years, constituted the control group. The mean age of the controls was 46.26 years (females, 47.45; males, 44.83). The study was approved by the Bioethics Committee at Poznań University of Medical Sciences (54/10, 17 June 2010).

Medical history acquisition, physical examination and additional tests were performed for all patients. Before enrolment, all patients and controls were informed about the nature and the aim of the study and gave their written informed consent. The diagnosis of rosacea was made on the basis of their medical history and physical examination. A standard classifications system, published by the National Rosacea Society (Wilkin et al., 2002), was used in the process of the diagnosis and classification of rosacea.

**Plasma samples.** Samples of peripheral blood (20 ml) from the cubital vein were collected from all study participants between 8 a.m. and 1 p.m. into EDTA tubes (Monovette, Sarstedt). The blood was centrifuged at 500 g for 10 min. The serum specimen was separated into three parts and stored at −80 °C.

**Preparation of bacterial protein for Western blotting of patient serum samples.** *B. oleronius* cells were cultured in nutrient broth to the stationary phase and subjected to cell surface protein extraction using 0.2 % (v/v) Triton X-100 as previously described (Lacey et al., 2007; O’Reilly et al., 2012a). The protein concentration was determined by Bradford assay and protein was resuspended at a concentration of 1 µg µl−1 in denaturing sample buffer. *Bacillus* protein (20 µg per well) was separated by 1D SDS-PAGE on 12.5 % acrylamide gels. Following electrophoresis, *Bacillus* proteins were transferred to nitrocellulose membranes which were sectioned into strips. Following a membrane blocking wash, individual serum samples (diluted 1/100 in antibody diluting buffer) were applied overnight at 4 °C. Following a TBS-Tween wash, the secondary anti-human IgG-HRP-linked whole antibody (Sigma) was applied at a dilution of 1/1000 for 2 h at room temperature. Immunoreactive protein bands were visualized by incubating membrane strips in diaminobenzidine tetrahydrochloride [DAB; 1 mg l−1 in 100 mM Tris/HCl (pH 7) containing 15 µl hydrogen peroxide] for 10 min at room temperature. All Western blots were performed using blinded serum samples and all were performed on three independent occasions.

**Standardized skin surface biopsy (SSSB).** One drop of cyanoacrylate adhesive was placed on a glass slide with a pre-marked square surface area of 1 cm². The slide was applied to the skin in the central area of the right cheek on a patient’s face. After 30 s, the slide was removed gently and one drop of immersion oil was added. A coverslip was placed on the sample and the specimen was examined under an optical microscope (magnified × 40 and × 100). The number of *Demodex* mites per cm² was enumerated by microscopic examination.

**Sebumetric test.** The level of sebum secretion by the skin was measured using a Sebumeter SM 815 Courage-Khazaka (Courage-Khazaka Electronic) as recommended by the manufacturer. A piece of 0.1 mm tape on a measuring probe, equipped with a spring to assure constant and even pressure, was placed on the skin of the centre of the patient’s chin for 30 s. The probe was then inserted into the sebumeter, where the amount of sebum on the surface of the skin was measured and expressed as µg cm⁻².

**Statistical methods.** The statistical significance was assessed by the χ²-test and Student’s t-test using GraphPad Prism version 5.00 for Mac OS X, GraphPad Software, www.graphpad.com. P-values <0.05 were considered statistically significant.

**RESULTS**

**Reactivity of patient sera to *Bacillus* proteins**

Protein was extracted from *B. oleronius* cells, resolved by 1D SDS-PAGE and transferred to membranes for Western blotting as described. Serum from patients with erythematotelangiectatic rosacea and controls was isolated and used to probe membranes containing the *Bacillus* proteins. The number of serum samples showing reactivity to the 62 and 83 kDa proteins of *B. oleronius* was calculated for each cohort (Fig. 1). The results revealed that 26.9 % (14/52) of controls showed reactivity to the bacterial proteins while 82.6 % (62/75) (*P*=0.0016) of patients diagnosed with erythematotelangiectatic rosacea showed reactivity to
the Bacillus proteins (Fig. 2). Rosacea patients could be divided into two groups on the basis of their reactivity (62/75) or non-reactivity (13/75) to the Bacillus proteins and were termed Bacillus protein reactive or Bacillus protein non-reactive, respectively.

**Analysis of Demodex population in rosacea patients and controls**

Analysis of the Demodex population in the skin of rosacea patients and controls revealed a statistically greater number of Demodex mites in the skin of rosacea patients that showed reactivity to the Bacillus proteins (P<0.0001) (Fig. 3). There was a slightly lower, although statistically non-significant (P=0.559), Demodex population in the skin of Bacillus protein non-reactive rosacea patients than in Bacillus protein reactive patients.

**Bacillus antigen reactive rosacea patients display reduced levels of sebum in their skin**

Analysis of the sebum level in the skin of patients and controls demonstrated that Bacillus protein reactive rosacea patients showed a lower level of sebum than the controls (P=0.0013) (Fig. 4). There was no significant difference between the sebum level in control and Bacillus protein non-reactive rosacea patient sera (P=0.548). Interestingly, the Bacillus protein reactive rosacea patient sera showed a significantly lower level of sebum than the antigen non-reactive rosacea patients (P=0.0159).

**DISCUSSION**

The results presented here indicate that sera from 82.6% of erythematotelangiectatic rosacea patients reacted with the 63 and/or 82 kDa protein(s) of *B. oleronius*. In addition, these patients displayed a higher population of Demodex mites in their skin and a lower level of sebum than controls.
uncover the significance of the increased density of mites on the facial skin of rosacea patients and their role in controls was measured and expressed as µg cm–2. Rosacea (+ive) indicates rosacea patients who were reactive to the Bacillus proteins; Rosacea (−ive) indicates rosacea patients who did not react to the Bacillus proteins. *P=0.0159, **P=0.0013.

Fig. 4. Variation in sebum level on skin of rosacea patients and controls. The sebum level on the skin of rosacea patients and controls was measured and expressed as µg cm–2. Rosacea (+ive) indicates rosacea patients who were reactive to the Bacillus proteins; Rosacea (−ive) indicates rosacea patients who did not react to the Bacillus proteins. *P=0.0159, **P=0.0013.

A possible role for micro-organisms in the aetiology of rosacea has been the subject of significant debate (Li et al., 2010; Jarmuda et al., 2012). Investigators have attempted to uncover the significance of the increased density of Demodex mites on the facial skin of rosacea patients and their role in the pathogenesis of the disease (Erbaçi & Ozgözaş, 1998; Yamasaki & Gallo, 2009). One of the suggested pathogenic mechanisms is connected with the fact that Demodex mites may transmit various bacteria. This theory is supported by the effectiveness of the antibiotic treatment (e.g. doxycycline, minocycline tetracycline), although these antibiotics may also function as anti-inflammatory agents (Gupta & Chaudhry, 2005).

*B. oleronius*, originally isolated from a *D. folliculorum* mite from a PPR patient, may play a role in the development of dermatological changes associated with rosacea (Lacey et al., 2007). Two proteins derived from *B. oleronius* (62 and 83 kDa) were isolated and their highly immunogenic properties were demonstrated. Exposure of neutrophils to *B. oleronius* proteins leads to their activation and triggers the release of matrix metallopeptidase 9 (MMP-9) and cathepsin D. The stimulation of IL-8 and TNF-α production may result in the development of an inflammatory process in vivo (O’Reilly et al., 2012a). The exposure of neutrophils to immunogenic proteins may occur in and around the pilosebaceous unit, when the proteins of *B. oleronius* are released from the dead Demodex mites (O’Reilly et al., 2012a). As a consequence of the inflammatory process, the structures of the tissue surrounding the pilosebaceous unit may be damaged (Kafienah et al., 1998).

The release of *B. oleronius* antigens may explain neutrophil activation around the pilosebaceous unit and the fact that the inflammatory process does not extend beyond this area. It is possible that exposure to low levels of Bacillus protein in normal skin does not sufficiently challenge the immune response but that the large amounts of material released within the pilosebaceous unit in rosacea patients may induce neutrophil migration and activation (O’Reilly et al., 2012a). Antibiotics that are commonly used in the treatment of rosacea do not reduce the population of Demodex but inhibit the growth of *B. oleronius* (Lacey et al., 2007) and thus may prevent the release of Bacillus-associated immunogenic proteins into and around the pilosebaceous unit. After antibiotic therapy is discontinued, rosacea symptoms may return, possibly due to the gradual revival of the *B. oleronius* population in the digestive system of the Demodex mites (Jarmuda et al., 2012).

The revival of the *B. oleronius* population is possible due to the fact that this bacterium, like other members of the Bacillus family, exists in two possible stages: vegetative and endosporic (Szkaradkiewicz et al., 2012). *Demodex* mites migrate on the surface of the host skin and most probably feed on the sebum components and epithelial cells. *B. oleronius* endospores enter their digestive systems in the process and germinate into their vegetative forms. PPR patients have higher pH and reduced levels of hydration of their facial skin (Ni Raghallaigh & Powell, 2009). They also display a different composition of fatty acids in the sebum, with elevated levels of myristic acids and reduced levels of specific saturated fatty acids (Ni Raghallaigh et al., 2012). Perhaps such conditions, not necessarily connected with the levels of sebum but rather with its composition, create a favourable environment for the development of the mite population.

While the aetiology of rosacea is unclear, dermatological, immunological, microbiological and environmental components probably contribute to its appearance (Gupta & Chaudhry, 2005; Yamasaki & Gallo, 2009). Changes to the skin such as an alteration in the amount and composition of sebum (Ni Raghallaigh et al., 2012) may favour the growth of the Demodex population. The elevated population of Demodex mites in the pilosebaceous unit of rosacea patients may physically distend the unit and facilitate the exit of bacterial proteins and toxins, which might leak into the surrounding tissue and attract neutrophils (O’Reilly et al., 2012a). This scenario might explain why inflammation is often centred on the pilosebaceous unit in rosacea. Treatment of rosacea with antibiotics destroys the Bacillus population within the Demodex mite and thus prevents the release of additional proteins. Once antibiotic therapy ends, the remaining mites may encounter Bacillus endospores on the skin, which germinate in their digestive tract and allow them to feed upon the altered sebum content of the face, thus leading to the renewal of the release of Bacillus proteins and the reappearance of symptoms.

Alterations in the nature of the sebum produced in the face may facilitate the increase in the density of Demodex in the
skin of rosacea patients. These may release bacterial antigens upon their death in and around the pilosebaceous unit, and the antigens of *B. oleronius* have been shown to induce an inflammatory reaction (O’Reilly et al., 2012a). This scenario would implicate bacteria as having a key role in the induction of rosacea, but this role might only come into play once other factors (e.g. vascular damage, altered sebum, increased *Demodex* density) have occurred. A clear understanding of the factors that contribute to the aetiology of rosacea will assist in the development of more effective therapies for the control of this chronic, disfiguring condition.

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