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Mite-related bacterial antigens stimulate inflammatory cells in rosacea

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Conflicts of interest
None declared.

Summary

Background Patients with papulopustular rosacea have a higher density of *Demodex folliculorum* mites on their faces than normal subjects but the role, if any, of their mites in initiating inflammation is disputed. Selective antibiotics are effective in reducing the inflammatory changes of papulopustular rosacea, but their mode of action is unknown.

Objectives To investigate whether a *D. folliculorum*-related bacterium was capable of expressing antigens that could stimulate an inflammatory immune response in patients with rosacea.

Methods A bacterium (*Bacillus oleronius*) was isolated from a *D. folliculorum* mite extracted from the face of a patient with papulopustular rosacea, and was investigated further.

Results This bacterium produced antigens capable of stimulating peripheral blood mononuclear cells proliferation in 16 of 22 (73%) patients with rosacea but only five of 17 (29%) control subjects ($P = 0{\cdot}0105$). This antigenic preparation was fractionated into 70 subfractions and the proteins in each fraction were visualized by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Western blot analysis revealed the presence of two antigenic proteins of size 62 and 83 kDa in fractions when probing with sera from patients with rosacea. No immunoreactivity to these proteins was recorded when probing with sera from control patients. Two-dimensional electrophoretic separation was used to isolate these proteins and matrix-assisted laser desorption/ionization time-of-flight analysis was employed to identify the relevant peptides. The 62-kDa immunoreactive protein shared amino acid sequence homology with an enzyme involved in carbohydrate metabolism and signal transduction while the 83-kDa protein was similar to bacterial heat shock proteins.

Conclusions Antigenic proteins related to a bacterium (*B. oleronius*), isolated from a *D. folliculorum* mite, have the potential to stimulate an inflammatory response in patients with papulopustular rosacea.

Papulopustular rosacea is a chronic inflammatory dermatosis of the convexities of the central face characterized by the presence of multiple small dome-shaped erythematous papules and papulopustules arising on a background of fixed inflammatory erythema.[1] The diagnostic criteria, classification and grading of rosacea have recently been outlined,[2,3] but its aetiology and pathogenesis are poorly understood.[4] The disease usually appears after the age of 30 years, is commoner in individuals with fair skin, and a positive family history of rosacea is present in up to 30% of patients.[5] Histopathological study of biopsies taken of papules and pustules from skin of patients with rosacea often shows a follicular-oriented inflammatory response,[6] and *Demodex folliculorum* mites are frequently seen in the follicles of biopsies from patients with rosacea.[7] Using the skin surface biopsy technique which extracts mites from follicular canals, several investigators have shown a significantly increased density of *D. folliculorum* mites in the facial skin of patients with rosacea when compared with control subjects,[8,9] but the relevance of this finding to the pathogenesis of the condition is

disputed. Papulopustular rosacea consistently responds to selected antibiotic therapy with clearing of inflammatory lesions, but the mechanism of action of these antibiotics is unknown, and the condition usually relapses within months of the antibiotic being discontinued.

In an attempt to reconcile the apparently disparate findings of increased numbers of *D. folliculorum* mites, perifollicular inflammation, and response of papulopustular rosacea to selective antibiotic therapy, we decided to investigate whether a *D. folliculorum*-related bacterium could be identified which might express stimulatory antigens capable of initiating an inflammatory response in patients with papulopustular rosacea.

A bacterium (*Bacillus oleronius*) cultured from a mite preparation in this study had been previously isolated from the hindgut of a termite.[10] This microorganism was investigated further.

Materials and methods

Extraction and dissection of mites

Following informed consent, *D. folliculorum* mites were extracted from designated facial sites of 40 patients with papulopustular rosacea using the skin surface biopsy technique with cyanoacrylate glue and glass slides as previously described.[8] The samples were studied microscopically at standard magnifications (× 40, × 100, × 400). One live mite from each patient sample was removed from the microscope slide and microdissected on nutrient agar using sterile fine forceps and dissecting tweezers.

Isolation of bacteria from facial skin swabs

Each patient with papulopustular rosacea had skin surface swabs were taken from the same designated facial areas as those from which *Demodex* mites were extracted. The swabs were streaked on to separate nutrient agar plates and incubated at 30 °C for 2 days to evaluate the microflora.

Isolation of bacteria from *Demodex folliculorum* preparations

The nutrient agar plates containing the microdissected mites were incubated at 30 °C for 2 days to achieve confluent bacterial growth. All cultures were sent to the National Collections of Industrial and Marine Bacteria (NCIMB), Aberdeen, U.K. for identification. The Kirby–Bauer test was used to test for antiobiotic sensitivity of cultured microorganisms.

Preparation of antigen

Bacillus oleronius was cultured in 250 mL nutrient broth (Oxoid, Basingstoke, U.K.) for 48 h at 30 °C and 180 r.p.m. Stationary phase cells were harvested by centrifugation (4000 ***g*** in a Beckman GS-6 centrifuge; Beckman Instruments, Palo Alto, CA, U.S.A.) and washed with phosphate-buffered saline (PBS, pH 7·2). Growth medium conditioned by the culture of *B. oleronius* ('supernatant') was diluted 1/10 with PBS and filter-sterilized for use in peripheral blood mononuclear cells (PBMC) proliferation assays. *Bacillus oleronius* cells were washed twice with PBS (pH 7·2) before resuspension in 2 mL of Break's buffer [10 mmol L^{-1} KCl, 3 mmol L^{-1} NaCl, 4 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} piperazine-N,N'-bis(2-ethanesulphonic acid); pH 7·2] plus Triton-X 100 (10% w/v) to solubilize outer membrane proteins. Protease inhibitors (10 μg mL^{-1} leupeptin, pepstatin A, aprotinin and N-α-*p*-tosyl-L-lysine chloromethylketone hydrochloride) were added. Cell suspensions (5 mL) were sonicated for 10 s using a Branson Soniprobe type 7532B (Dawe Instruments, London, U.K.) to dislodge antigens associated with the bacterial cell wall. Cells were filtered off and the 'sonicate' preparation was stored at −20 °C.

Peripheral blood mononuclear cells proliferation assay

Peripheral blood (5 mL) was obtained from 22 patients with rosacea (nine women and 13 men, age range 32–64 years) and age- and sex-matched control volunteers. PBMC were prepared for the proliferation assay as described previously.[11,12] A proliferation index > 2 was considered positive in accordance with the literature.[11–13] All assays were performed on three independent occasions and the data were statistically analysed using the Sigma-Stat Statistics Package using Fisher's exact test.[14]

Antigen fractionation by Q-Sepharose charge separation

The proteins in the 'Triton-X' preparation were purified and chromatographed by Q-Sepharose charge separation using an ÄKTA Purifier 100 system (Amersham Biosciences, Amersham, U.K.). The starting material was loaded on to Q-Sepharose (1·5 × 2 cm, 1 mL min^{-1}, 1 mL fractions collected, eluted with a 30 mL linear gradient of 0·5 mol L^{-1} NaCl in Break's buffer). Peak fractions containing the antigens of interest were identified by immunoreactivity against sera of patients with rosacea.

Western blot analysis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 12·5% (w/v) polyacrylamide gel in a discontinuous buffer system and subject to electrophoresis at 200 V for 8 h. For Western blot analysis the primary antibody consisted of pooled sera from six patients with rosacea or controls, diluted 1/150 with antibody-diluting buffer [3% (w/v) bovine serum albumin, 10% (w/v) nonfat dried milk dissolved in TBS-T (78·8 g Tris-HCl, 87·65 g NaCl and 500 μL Tween-20 in 1 L H_2O)] prior to use. The secondary antibody was antihuman IgG-horseradish peroxidase-linked whole antibody (Sigma Aldrich Chemical Co. Ltd, Poole, U.K.) which was diluted 1/1000 with the antibody-diluting buffer prior to addition to the

membrane. In all cases duplicate gels [SDS-PAGE and two-dimensional (2D)] were prepared under identical conditions, one for immunoblotting and the other for Coomassie staining.

Two-dimensional gel electrophoresis and immunoblot analysis of antigenic fractions to IgG

Isoelectric focusing and 2D electrophoresis of fractionated antigenic protein samples was performed as described previously.[15] Western blot analysis was carried out as above.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

To carry out peptide analysis, protein spots of interest were excised from 2D gel (Coomassie stained) and diced finely. Gel pieces were prepared for matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) analysis which was performed as described.[15] Mass spectra were recorded using an Ettan MALDI-ToF spectrometer (Amersham Biosciences, Freiburg, Germany), analysed using MALDI evaluation software (Amersham Biosciences), and proteins were identified using the PMF Profound search engine for peptide mass fingerprints.

Ethical approval

Ethical approval for this study was granted by the Research Ethics Committee of the Mater Misericordiae University Hospital, Dublin, Ireland (ref. 1/378/638) and informed consent was required from each participant.

Results

Extraction and dissection of mites

Mites were extracted from the following skin areas: the forehead, cheek, chin or nose of 40 patients with papulopustular rosacea using the standardized skin surface biopsy.[8] After 48 h of incubation, bacterial colonies were observed growing around the site of the microdissected mites. Only bacterial colonies with different phenotypes were extracted aseptically from each agar plate, streaked on to new plates and incubated again at 30 °C for 2 days.

Isolation of bacteria from facial skin swabs

After 48 h of incubation, bacterial colonies were observed growing where the facial swab had been streaked on the plates. The phenotypically different colonies were inoculated on to new agar plates and sent to the NCIMB for identification. Bacteria isolated from facial swabs included *Micrococcus agilis*, *M. luteus*, *Micromonospora halophytica*, *Staphylococcus simulans*, *S. capitis*, *S. epidermis*, *Haloanella gallinarum*, *Pantoea agglomerans* and *Dietzia maris*.

Isolation of bacteria from *Demodex folliculorum* preparations

One bacterial species, cultured as an orange colony from one microdissected *Demodex* mite from a patient with papulopustular rosacea, appeared distinctive. This bacterium was identified to species level at the NCIMB by analysis of the 16s rDNA sequence, as an endospore-forming bacterium, *B. oleronius*, which had previously been found in association with the midgut of termites and identified as Gram negative.[10] Antibiotic sensitivity testing showed that *B. oleronius* was sensitive to tetracycline, doxycycline and minocycline. *Bacillus oleronius* was thus selected for further investigation.

Preparation of antigen

Bacillus oleronius supernatant and sonicate antigen preparations were produced as described above and subsequently used in the experiments outlined below. Although this *Bacillus* could be cultured at both 37 °C and 30 °C, culturing of this bacterium at 30 °C rather than 37 °C induced the expression of increased levels of the stimulatory antigens that were the focus of this work.

Peripheral blood mononuclear cells proliferation assay

Bacillus oleronius 'sonicate' and 'supernatant' preparations were applied to PBMC from patients with papulopustular rosacea or controls. The results (Table 1) demonstrate that the *B. oleronius* 'sonicate' preparation activated PBMC proliferation in 16 of 22 (73%) patients with rosacea and five of 17 (29%) controls. Using Fisher's exact test,[14] the results show statistical significance with $P = 0{\cdot}0105$ ($P < 0{\cdot}05$) (d.f. = 1). In contrast, the *B. oleronius* 'supernatant' preparation activated PBMC in one of nine (11%) patients with papulopustular rosacea and six of 17 (35%) controls – a result which was not statistically significant ($P = 0{\cdot}357$) (Table 1).

Antigen fractionation by Q-Sepharose charge separation

In order to identify the specific antigen(s) responsible for the differential reactivity observed above, fractions collected corresponding to the protein peaks on the chromatograph (Fig. 1a) were analysed by SDS-PAGE, and Western blot analysis was performed. Fractions 12–56 were analysed by SDS-PAGE and fractions 21–37 showed the greatest number and intensity of bands (Fig. 1b, c). Fractions 21–29 showed an increase in the number of protein bands ranging in size from 16·5 to 83 kDa (Fig. 1b). A decrease in the number of protein bands was observed in fractions 30–37 (Fig. 1c), with a consistent decrease across fractions 38–54 (data not presented).

Western blot analysis

Immunoblot analysis of the above SDS-PAGE gels, using pooled sera from six patients with papulopustular rosacea as the

Antigen preparation	Positivity in patients with rosacea, n (%)	Positivity in controls, n (%)	P-value
B. oleronius sonicate	16/22 (73%)	5/17 (29%)	0·0105 ($P < 0·05$)
B. oleronius supernatant	1/9 (11%)	6/17 (35%)	0·3574 ($P > 0·05$)

Table 1 Response of peripheral blood mononuclear cells from patients with rosacea and controls following challenge with *Bacillus oleronius* antigen preparations

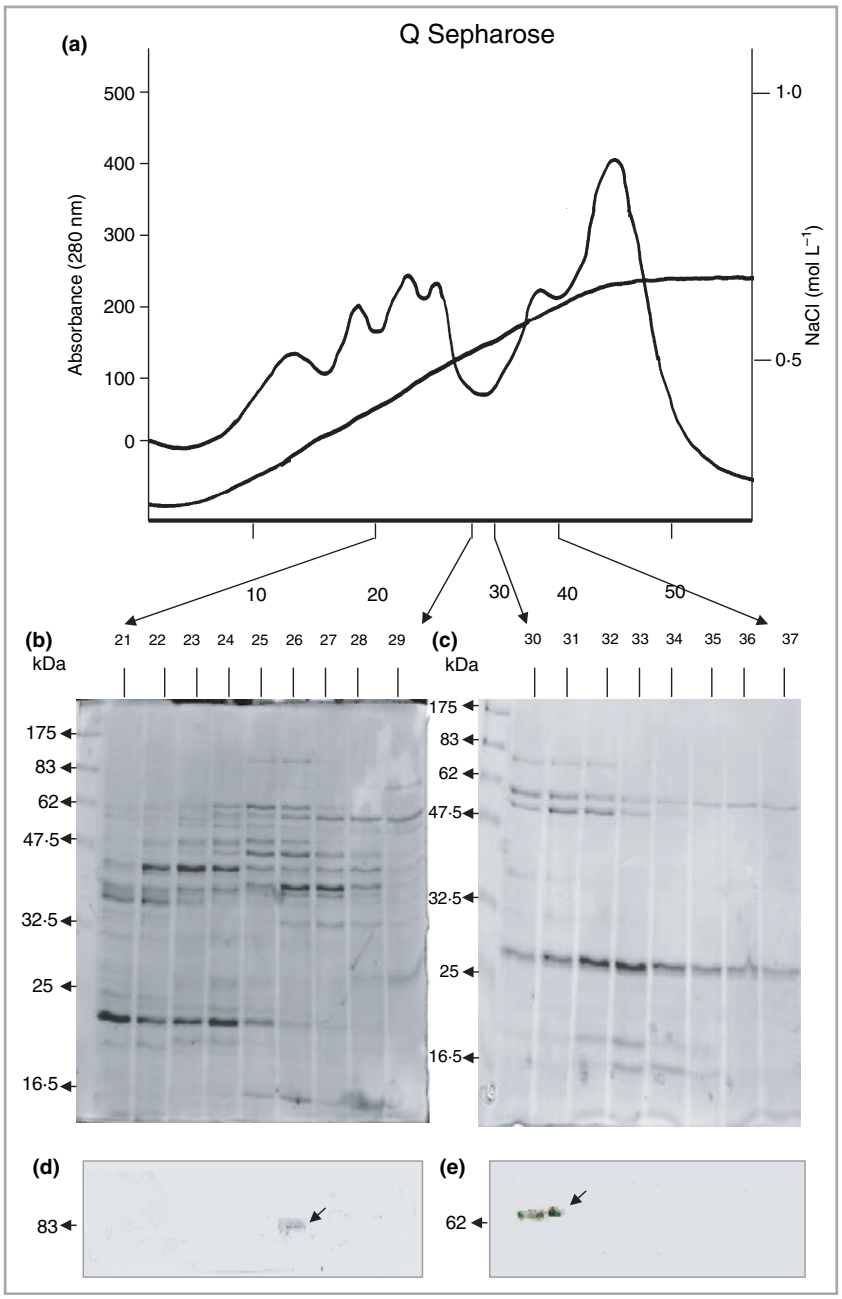

Fig 1. (a) Chromatograph of *Bacillus oleronius* 'sonicate' preparation. Protein peaks correlate with proteins in fractions. (b) Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of fractions 21–29. (c) SDS-PAGE analysis of fractions 30–37. (d) Western blot analysis of fractions 21–29, probing with pooled rosacea patient sera. Reactivity was visualized in fraction 26 to the 83-kDa protein. (e) Western blot analysis of fractions 30–37, probing with pooled rosacea patient sera. Reactivity was visualized in fractions 30 and 31 to the 62-kDa protein.

primary antibody and antihuman IgG-horseradish peroxidase-linked whole antibody as the secondary antibody, confirmed that sera from patients with rosacea contained antibodies to proteins of size 83 kDa present in fraction 26 (Fig. 1d) and 62 kDa present in fractions 30 and 31 (Fig. 1e). No other immunoreactive antigens from *B. oleronius* were visualized in these blots.

Two-dimensional gel electrophoresis immunoblot analysis of antigenic fractions to IgG

2D gel electrophoresis was performed on fractions 26, 30 and 31 to isolate the proteins of interest prior to further characterization by mass spectrometry. The samples were primarily separated according to their isoelectric point, across a pH gradient, followed by separation due to molecular size on an SDS-PAGE gel. 2D gels showed more than 30 reproducible protein spots from fraction 26 (Fig. 2a), 25 from fraction 30 (Fig. 2b) and 18 from fraction 31 (Fig. 2c). The 83-kDa protein from fraction 26 (Fig. 2a) was shown to consist of two proteins of different pI values (5·35 and 5·5). Following immunoblot analysis of this 2D gel (as previously described), sera from patients with rosacea showed reactivity only towards one of these proteins at pI 5·5 (Fig. 2a). The 62-kDa protein was shown to consist of one large protein (pI 5·0), to which the sera of patients with rosacea was strongly reactive (Fig. 2b, c).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

The spots visualized by Coomassie staining (Fig. 2a–c), corresponding to those that were immunoreactive with rosacea patient antibodies by Western blot analysis, were prepared for MALDI-ToF analysis. From the MALDI-ToF spectra of spot 1 (83 kDa, pI 5·5), spot 2 (62 kDa, pI 5·0) and spot 3 (62 kDa, pI 5·0), peptide masses were used for searches in the National Center for Biotechnology Information (Bethesda, MD, U.S.A.) database. The parameters were as follows: enzyme, trypsin; maximum missed cleavage, 1; peptide mass tolerance, 1 Da; monoisotopic masses tolerance, 1 Da. In the tryptic digest of the 83-kDa protein spot that evoked reactivity from patient sera, protein coverage with other candidates on the database ranged from 8% to 18%. Results include a 9% protein coverage with an 85-kDa heat shock-like protein (Z score = 1·54) and an 18% coverage with an NADP-dependent malic enzyme (Z score = 1·29) (Table 2). The 62-kDa spots (spots 2 and 3) also produced an antibody response, and revealed protein similarity with other candidates on the database ranging from 9% to 21%. Candidate functions included phosphorylation, protease activity, enzyme activity and surface adhesion protein.

Discussion

Demodex mites are inhabitants of normal adult human skin whose life-cycle lasts about 14 days.[16] While they were first described as early as 1842 their role in the biology of the skin has received little attention from dermatologists. As the vast majority of individuals experience no adverse reaction from the presence of these mites, it is likely that they either avoid exposure to host immune defences or have the ability to downregulate host immunity so that they can survive in the cutaneous environment of their human host. *Demodex*

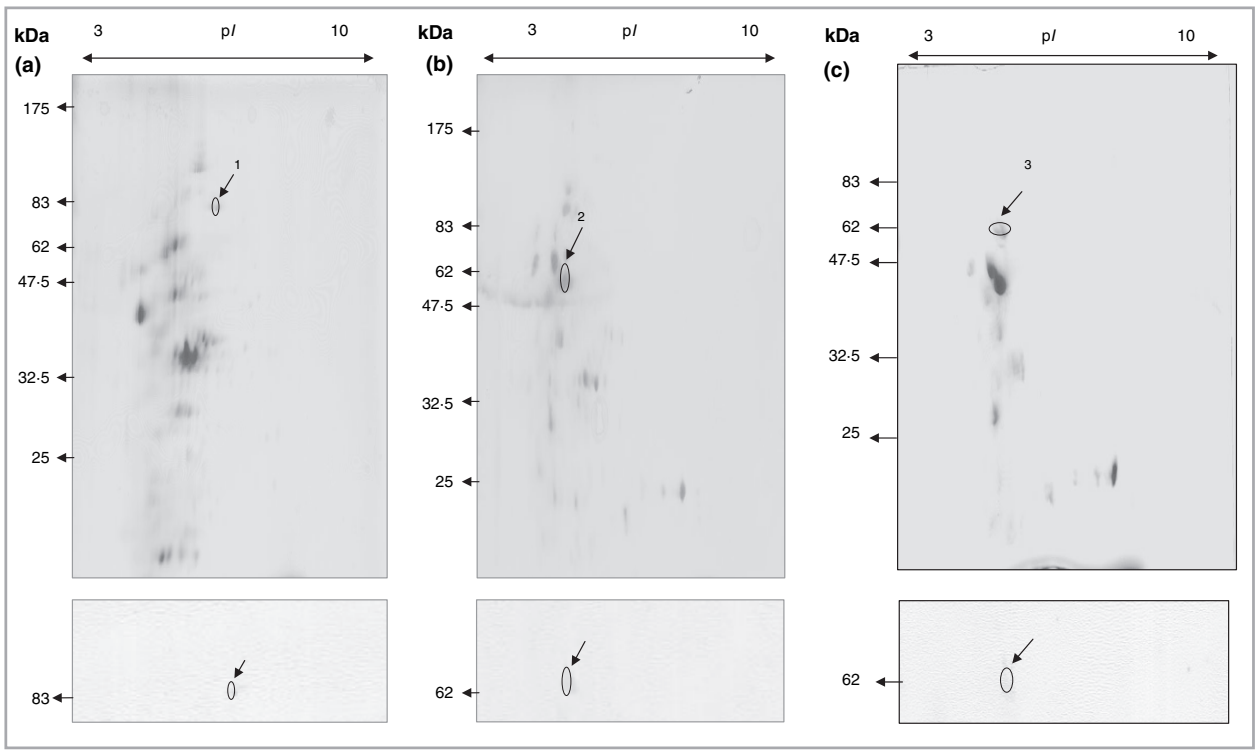

Fig 2. Two-dimensional gel and Western blot analysis of (a) antigenic fraction 26 (83-kDa protein), (b) antigenic fraction 30 (62-kDa protein) and (c) antigenic fraction 31 (62-kDa protein).

Table 2 Comparison of 62-kDa and 83-kDa antigens with proteins in the National Center for Biotechnology Information (NCBI) database

Function	Identified proteins	Spot number	NCBI accession number	% protein coverage	pI value	Z score
62-kDa antigen						
Rapid utilization of carbohydrates/signal transduction	Phosphoenolpyruvate-protein phosphotransferase	2	NP_975272.1	9	5·4	1·79
Catalyses the synthesis of major membrane phospholipid	Putative cardiolipin synthetase	3	NP_269350.1	16	6·1	1·39
Hydrolysis and inactivation of penicillin	Metallo-beta-lactamase superfamily protein	2	AA082612.1	21	5·8	1·34
Protease activity	Hypothetical protein	2	CAG20611.1	13	6·0	1·32
Enzyme	Major catalase in spores	3	NP_391742.1	10	5·5	0·53
Gene encoding class of surface proteins (lipoproteins)	Adhesin pMGA1.4	3	AAF91415.1	18	5·3	0·49
83-kDa antigen						
Molecular chaperone (stress response)	Heat shock-like 85-kDa protein	1	HS85_TRYCR	9	5·1	1·54
Metabolic enzyme/adherence molecule	NADP-dependent malic enzyme	1	NP_422343.1	18	5·2	1·29
Molecular chaperone (stress response)	Small heat shock-like protein Hsp3	1	NP_280056.1	8	5·5	0·30

folliculorum is usually found in the upper canal of the pilosebaceous follicle and often there are several mites in a single follicle, with mouthparts directed towards the fundus.[17] The role of *Demodex* mites in the production of cutaneous inflammation is disputed, but *Demodex* mites have been previously shown to act as vectors for the transportation of microbes on the face[18] and some authors attribute specific facial eruptions to these organisms.[19] Patients with rosacea have a higher density of *D. folliculorum* mites in the areas of facial skin affected by the condition compared with subjects with normal skin, as demonstrated by the skin surface biopsy technique which extracts these mites from the follicular canal and allows their quantification.[8,9]

The mechanism by which antibiotics successfully clear the inflammatory lesions of papulopustular rosacea is unknown. It has been suggested that antibiotics work through anti-inflammatory mechanisms, but other potent anti-inflammatory drugs are ineffective and immunosuppressive agents such as steroids (administered topically or systemically) and tacrolimus can make the inflammatory eruption of rosacea worse.[20]

The fact that only selective antibiotics are effective in rosacea suggested to us the possibility that a bacterial agent may be involved in the pathogenesis of the disease. A *D. folliculorum*-related bacterium sensitive to the antibiotics used in the treatment of rosacea could explain the induction of inflammatory changes in papulopustular rosacea. Such changes would be centred on the centrofacial pilosebaceous follicles which are highly populated with these mites in papulopustular rosacea. Appropriate antibiotic treatment could reduce the bacterial-induced inflammatory response and possibly affect the viability of mites, with clinical relapse occurring either when the bacterial antigen load increased again after cessation of treatment or when the follicle again became distended with a repopulation of multiple mites causing 'leakage' of bacterial antigens through follicular walls.

Following analysis of the microorganisms cultured from swabs and skin surface biopsies, we decided to study in detail *B. oleronius* because this microorganism has previously been reported as a member of the hindgut flora of the termite *Reticulitermes santonensis*[10] and is not a recognized human cutaneous commensal nor a likely contaminant of samples. This *Bacillus* is an endospore-forming bacterium previously identified as being Gram negative, a finding supported by our Gram stain method. However, this bacterium has contradictory characteristics, sharing Gram-positive cell wall components like all *Bacillus* species.[10] *Bacillus oleronius* demonstrated sensitivity to several antibiotics which are effective in the treatment of rosacea (tetracycline, doxycycline and minocycline). Exposure of PBMC from patients with papulopustular rosacea to the 'sonicate' preparation fractions containing antigenic components of *B. oleronius* resulted in significantly more PBMC stimulation (73%) than PBMC from control patients (29%) which suggests prior sensitization of PBMC from patients with rosacea to these surface bacterial antigens. Characterization of the bacterial antigens by SDS-PAGE and Western blot analysis revealed two specific antigenic proteins (83 kDa and 62 kDa) which were immunoreactive. Further characterization by MALDI-ToF analysis showed the 83-kDa protein to have homology with heat shock proteins, while the 62-kDa protein shared amino acid sequence homology with a protease enzyme involved in carbohydrate metabolism and signal transduction. Both these types of antigenic proteins have the potential to be involved in the immune-based inflammatory cascade.

The significance of the finding of *B. oleronius* in association with *D. folliculorum* mites and its role, if any, in biological functioning of these mites needs to be further defined. The

possible presence of other mite-related bacteria also needs to be explored as this *Bacillus* may represent only one of several different or even closely related species or subspecies present in these mites. It has been shown that several such related bacteria can be identified sharing a niche by multiple culture conditions and by 16s rDNA analysis.[21] *Bacillus oleronius* was isolated from one microdissected *D. folliculorum* mite from one patient with rosacea. Our failure to isolate this *Bacillus* from mites extracted from other patients with rosacea is puzzling, and may be related to previous antibiotic treatment of some of the patients with rosacea prior to sampling which may have eradicated or inhibited the growth of the bacterium. Another possible reason for our inability to culture this *Bacillus* from mites extracted from other patients is the questionable reliability of the traditional culture techniques we used. Other studies[21,22] have shown that these culture techniques do not select for all bacteria. By culturing our primary facial swab samples and microdissected mites at 30 °C we may have reduced the diversity of microflora retrieved. Subculturing *B. oleronius* at both 30 °C and 37 °C showed that the *Bacillus* grew more actively at the latter temperature. However, *B. oleronius* grown at 30 °C was used in the experiments described as this condition favoured expression of the antigens investigated. Future investigations of the flora of *D. folliculorum*-related bacteria should incorporate cultivation at 30 °C and 37 °C in both aerobic and anaerobic conditions as well as 16s rDNA analysis on whole mites.

Bacillus oleronius may have a symbiotic relationship with *Demodex* mites as is suggested by the findings of this *Bacillus* as a member of the hindgut flora of the termite *R. santonensis*. Such symbiotic associations between microorganisms and higher eukaryotes are common, and range from mutualistic (beneficial) to commensal (neutral) and parasitic (harmful).[23] Such symbiotic microorganisms can have clinical importance as therapeutic targets or by exposure of the host to infection. A recent report has shown that doxycycline treatment of patients with filariasis resulted in a marked reduction in the worm population through the antibiotic effect on its endosymbiotic *Wolbachia* bacteria,[24] while suppurative cutaneous reactions have been reported to gut-related symbiotic bacteria of leeches (*Hirudo medicinalis*).[25] It is possible that *B. oleronius* has a symbiotic relationship with *Demodex* mites similar to *Wolbachia* endosymbionts which facilitate embryogenesis and fertility in filarial worms. It has been repeatedly shown that patients with rosacea have increased facial *Demodex* mite populations. The large numbers of dying mites in the follicles of patients with rosacea may increase released bacterial antigen load to critical levels. The presence of multiple mites (both dead and alive) in individual follicles could distend or damage the follicular integrity to an extent that allows diffusion of mite-related bacterial antigens through the follicular wall, triggering a perifollicular host immune response.

Selective antibiotics are effective in clearing papulopustular rosacea but their mode of action is unknown. This work shows that consideration must be given to their potential to affect microorganisms such as *B. oleronius* or other follicular or mite-related bacteria. By reducing the antigenic load of these microorganisms or altering the numbers or biological functioning of *D. folliculorum* mites they may consequently affect the inflammatory changes which form the characteristic clinical features of the disease we recognize as papulopustular rosacea.

Acknowledgments

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References

1 Powell FC. Rosacea. *N Engl J Med* 2005; **352**:793–803.
2 Wilkin J, Dahl M, Detmar M *et al.* Standard classification of rosacea: Report of the National Rosacea Society Expert Committee on the Classification and Staging of Rosacea. *J Am Acad Dermatol* 2002; **46**:584–7.
3 Wilkin J, Dahl M, Detmar M *et al.* Standard grading system for rosacea: Report of the National Rosacea Society Expert Committee on the Classification and Staging of Rosacea. *J Am Acad Dermatol* 2004; **50**:907–12.
4 Powell FC. What's going on in rosacea? *J Eur Acad Dermatol Venereol* 2001; **14**:351–5.
5 Rebora A. The red face: rosacea. *Clin Dermatol* 1993; **11**:225–34.
6 Powell FC. Rosacea and the pilosebaceous follicle. *Cutis* 2004; **74**:9–12.
7 Aroni K, Tsagroni E, Lazaris AC *et al.* Rosacea: a clinicopathological approach. *Dermatology* 2004; **209**:177–82.
8 Bonnar E, Eustace P, Powell FC. The *Demodex* mite population in rosacea. *J Am Acad Dermatol* 1993; **28**:443–8.
9 Forton F, Seys B. Density of *Demodex folliculorum* in rosacea: a case control study using standardized skin surface biopsy. *Br J Dermatol* 1993; **128**:650–9.
10 Kuhnigk T, Borst EM, Breunig A *et al. Bacillus oleronius* sp. nov., a member of the hind-gut flora of the termite *Reticulitermes santonensis* (Feytaud). *Can J Microbiol* 1995; **41**:699–706.
11 von Poblotzki A, Gerdes C, Reischl U *et al.* Lymphoproliferative responses after infection with human parvovirus B19. *J Virol* 1996; **70**:7327–33.
12 Yokote R, Yagi H, Furukawa F, Takigawa M. Regulation of peripheral blood mononuclear cell response to *Dermatophagoides farinae* by substance P in patients with atopic dermatitis. *Arch Dermatol Res* 1998; **4**:191–7.
13 Sanni LA, Alsopp CE, Reubasaet L *et al.* Cellular response to *Plasmodium falciparum* erythrocyte membrane protein-1: use of relatively conserved synthetic peptide pools to determine CD4 T-cell responses in malaria-exposed individuals in Benin, West Africa. *Malaria J* 2002; **26**:7–12.
14 Wardlaw AL. *Practical Statistics for Experimental Biologists*. Chichester: Wiley, 2000.
15 Bergin D, Reeves EP, Renwick J *et al.* Superoxide production in haemocytes of *Galleria mellonella* – identification of proteins homologous to the NADPH oxidase complex of human neutrophils. *Infect Immun* 2005; **73**:4161–73.
16 Spickett SG. Studies on *Demodex folliculorum* Simon (1942). Life history. *Parasitology* 1961; **51**:181–92.
17 Desch C, Nutting WB. *Demodex folliculorum* (Simon) and *D. brevis* (Akulatova) of man; redescription and reevaluation. *J Parasitol* 1972; **58**:169–77.

18 Wolf R, Ophir J, Avigad J. The hair follicle mites (*Demodex* sp.). Could they be vectors of pathogenic micro-organisms? *Acta Derm Venerol (Stockh)* 1988; **68**:535–7.
19 Forton F, Germaux MA, Brasseur T *et al.* Demodicosis and rosacea: epidemiology and significance in daily dermatology practice. *J Am Acad Dermatol* 2005; **52**:74–87.
20 Antille C, Saurat JH, Lubbe J. Induction of rosaceiform dermatitis during treatment of facial inflammatory dermatoses with tacrolimus ointment. *Arch Dermatol* 2004; **140**:457–60.
21 Kroes I, Lepp PW, Relman DA. Bacterial diversity within the human subgingival crevice. *Proc Natl Acad Sci USA* 1999; **96**:14547–52.
22 Nelson KE, Zinder SH, Hance I *et al.* Phylogenetic analysis of the microbial populations in the wild herbivore gastrointestinal tract: insights into an unexplored niche. *Environ Microbiol* 2003; **5**:1212–20.
23 Werren JH. *Wolbachia* run amok. *Proc Natl Acad Sci USA* 1997; **94**:11154–5.
24 Taylor MJ, Mahunde WH, McGarry HF *et al.* Macrofilaricidal activity after doxycycline treatment of *Wuchereria bancrofti*: a double-blind randomized placebo-controlled trial. *Lancet* 2005; **365**:2116–21.
25 Steer A, Daley AJ, Curtis N. Suppurative sequelae of symbiosis. *Lancet* 2005; **385**:188.

