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Different pro-inflammatory and immunogenic potentials of *Propionibacterium acnes* and *Staphylococcus epidermidis*: implications for chronic inflammatory acne

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

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Summary

Introduction:

Propionibacterium acnes (PA) and *Staphylococcus epidermidis* (SE) are two major bacterial strains isolated from acne lesions. Nevertheless, only PA seems to be implicated in the pathogenesis of inflammatory acne vulgaris. Evidence for this, however, remains indirect and the precise role of PA in inflammatory acne is still a matter for conjecture. The aim of this study was to compare some pro-inflammatory and adjuvant properties of PA and SE.

Materials and Methods:

To determine some of the pathogenic, immunostimulatory, and pro-inflammatory properties of PA and SE, two experimental models of inflammation were used. *In vivo*; chronic inflammation was induced by intradermal injection of living bacteria into the ear. *In vitro*; peritoneal macrophages elicited by the bacteria were examined for their ability to generate reactive oxygen species (ROS), nitric oxide (NO), and cytokines.

Results:

PA, but not SE, evoked mild local inflammation of infected ears. Macrophages elicited with PA produced more tumor necrosis factor α and interleukin IL-12 than those induced with SE, while SE was a stronger inducer of IL-10 production. Both bacteria equally induced the generation of NO and ROS. In contrast, only PA showed adjuvant properties.

Conclusions:

The results of these studies indicate that SE, in contrast to PA, does not exert pro-inflammatory properties. Thus it is unlikely that SE may be implicated in the pathogenesis of inflammatory acne vulgaris.

Key words:

acne vulgaris • *Propionibacterium acnes* • *Staphylococcus epidermidis* • inflammation • TNF- α • IL-10 • ROS

Abbreviations:

OVA – ovalbumin, BSA – bovine serum albumin, HRP – horseradish peroxidase, OPD – o-phenylenediamine dihydrochloride, LCL – luminal-dependent chemiluminescence, HBSS – Hank's balanced salt solution, ROS – reactive oxygen species, PA – *Propionibacterium acnes*, SE – *Staphylococcus epidermidis*, TLR2 – Toll-like receptor 2, NO – nitric oxide, TauBr – taurine bromamine, PBS – phosphate-buffered saline solution.

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INTRODUCTION

Acne vulgaris is the most common cutaneous disorder and is of an etiology that appears to be multifactorial^{8, 13}. The four main etiological factors are: hypercornification of the pilosebaceous duct, increased sebum production, colonization by skin bacteria and, subsequently, the production of inflammation. Although acne lesions are mainly colonized by two genera of Gram-positive bacteria, *Propionibacterium* and *Staphylococci*, the most likely organism to blame is *Propionibacterium acnes* (PA)^{5, 8, 14}. Several lines of evidence have implicated PA in the pathology of acne lesions; nevertheless, the molecular mechanism by which PA induces inflammation is poorly understood^{11, 14}. It has been shown that PA, formerly known as *Corynebacterium parvum*, displays a variety of immunomodulatory properties. PA is a strong stimulant of pro-inflammatory mediators released by phagocytic cells, especially of tumor necrosis factor TNF- α ^{4, 22}. Additionally, this organism is known as an adjuvant for the reticuloendothelial system and as a stimulant of natural killer cells^{6, 19}. The current interest in PA revolves around whether its immunopotentiating properties are pertinent in the pathogenesis of acne and what the relative contributions of other skin bacteria are, especially *Staphylococcus epidermidis* (SE), to the pathology of inflammatory lesions³.

The aim of this study was to evaluate the potential contributions of two major bacteria strains of the skin in mediating acne inflammation. In order to determine some of the pathogenic, immunostimulatory, and pro-inflammatory properties of PA and SE, two animal models of inflammation were used: 1) an acute inflammation (peritonitis), induced by intraperitoneal injection of tested bacteria, and 2) a chronic skin inflammation, partially relevant to acne vulgaris, induced by intradermal injection of the bacteria into the ears of mice.

MATERIALS AND METHODS

Bacterial strains

PA was isolated from acne lesions of patients with mild acne vulgaris. For the present study we selected (PA) no. 496, a strain which belongs to biotype 1 (sorbitol+erythritol+ribose+), the most common biotype of *Propionibacterium* isolated from acne patients¹³. SE was isolated from normal human skin.

The selected micro-organisms were lyophilized and stored until used. To prepare a culture of the given strain for the investigations, the lyophilized material

was revived and inoculated onto the following media: PA on Scharf's Agar Base (Difco, USA), cultured in anaerobic conditions at 35°C for 72 h and SE on Tryptic Soy Broth (Difco, USA), cultured in anaerobic conditions at 35°C for 18 h. Following incubation, the culture was immediately cooled to 4°C and an aliquot was removed and quantitatively plated to determine cell number and verify purity. The remainder of the culture was centrifuged at $5,000 \times g$ for 10 min. The bacterial pellet was washed twice in 0.9% NaCl and finally suspended in 0.9% NaCl to get a suspension of a density of 5×10^9 colony-forming unit (CFU)/ml. The final bacterial suspension was incubated at 80°C for 30 min to heat-kill the bacteria and stored at 4°C until use. When living bacteria were necessary for the experiments, the suspension was prepared on the scheduled day of experiment and used immediately.

Mice

Inbred CBA/J female mice (8–12 weeks of age, 18–22 g) were maintained at the Animal Breeding Unit, Department of Immunology, Jagiellonian University Medical College, Cracow. All mice were housed 4–5 per cage in a laboratory room with water and standard diet *ad libitum*. The appropriate permission for using mice in this project was obtained from the local ethics committee.

Bacteria-induced skin inflammation

To determine the capacity of PA and SE to induce a local skin inflammatory response *in vivo*, we used the animal model of inflammation considered to be partially relevant to acne²⁷. The inflammation was evoked by intradermal injection of 20 μ l aliquots of 10^8 living bacteria in the central, ventral portion of the left ears of mice. As a control, 20 μ l aliquots of 0.9% NaCl solution was injected intradermally into the right ear. The mice were infected with either PA or SE. The observations were carried out for 3–4 weeks. Ear thickness was measured using engineering micrometers (Mitutoyo, Japan). Moreover, the mice were examined visually for the appearance of symptoms of local inflammatory reaction in both ears. There were two separate “blind” examiners. The evaluation scale of severity of inflammation consisted of 4 grades: 1) 0: no lesions, 2) +: ear thicker than the reference ear, 3) ++: ear thicker and reddened, 4) +++: purulent lesions within the ear, erosion.

Experimental model for testing adjuvant properties of PA and SE

Mice were immunized intradermally with ovalbumin (OVA, 3 mg/mouse; Sigma, USA) alone or OVA

together with heat-killed bacteria was injected at a dose of 5×10^8 /mouse. The mice were immunized twice at a 14-day interval. The blood serum was collected and the level of anti-OVA IgG antibodies was evaluated by ELISA. Each experimental group consisted of 15 mice.

Measurement of serum anti-OVA IgG antibody titers

Mice were anesthetized and bled on days 14 and 21 after primary immunization. Individual serum samples were stored at -80°C until used. The serum level of antibody against OVA was measured using a standard ELISA assay. Briefly, microtiter plates (Corning, USA) were coated overnight with $5 \mu\text{g/ml}$ of OVA (Sigma, USA) in phosphate-buffered saline (PBS; Sigma, USA) at 4°C . Non-specific binding was blocked with 4% bovine serum albumin (BSA; Sigma, USA) in PBS at room temperature for 1 h. Diluted serum samples (in 1% BSA in PBS) were added and incubated for 1 h at room temperature. The plates were then incubated with biotinylated goat anti-mouse IgG antibody (Sigma, USA) for 45 min at room temperature. Horseradish peroxidase (HRP) conjugated streptavidin diluted 1:1000 in 1% BSA/PBS was added and the plates were incubated for 45 min at room temperature. Then o-phenylenediamine dihydrochloride (OPD; Sigma, USA) was used as a substrate (5 mg of OPD in 10 ml of phosphate-citrate buffer, pH 5.0) and incubated with $40 \mu\text{l}$ of 30% H_2O_2 for 30 min at room temperature. The reaction was stopped with 3 M H_2SO_4 . Optical density was measured at 492 nm .

Induction of acute peritonitis

In order to induce acute inflammatory response, mice were injected intraperitoneally with either zymosan or heat-killed tested bacteria. Peritoneal exudate cells were used as a source of macrophages for *in vitro* studies.

Macrophages

Peritoneal mouse macrophages were induced by intraperitoneal injection of 1.0 ml of either zymosan A (1 mg/mouse ; Sigma, USA) or with heat-killed bacteria (PA, SE, 1×10^8 CFU/mouse). Cells were collected 24 h later by washing out the peritoneal cavity with 5 ml of PBS containing 5 U heparin/ml (Polfa, Poland). The cells were centrifuged and red blood cells were lysed by osmotic shock using distilled water; osmolarity was restored by adding twice concentrated PBS. The presence of macrophages (85–90%) was judged by cytochemical demonstration of non-specific esterase-positive mononuclear cells using α -naphthyl acetate (Sigma, USA).

Chemiluminescence assay of reactive oxygen species: luminol-dependent chemiluminescence

Heat-killed bacteria at a concentration of 1×10^9 CFU/ml or opsonized zymosan (as a positive control, 0.5 mg/well) were placed in 96 flat-bottom black plates (Nunc, Denmark) in Hank's Balanced Salt Solution (HBSS; Sigma, USA) and incubated at 4°C for overnight. Then, the unbound bacteria were washed out and freshly isolated peritoneal exudate cells (5×10^5 cells/well) suspended in HBSS plus luminol ($200 \mu\text{l}$) were added. Immediately after the addition of cells, photon emission was measured for 60 min on a Lucy 1 luminometer (Anthos, Austria). Each experiment was run in duplicate.

Cell culture. Activation of cells for production of inflammatory mediators

Macrophages were cultured in 24-well flat-bottom cell culture plates at 5×10^5 cells/well in RPMI 1640 medium (JR Scientific Inc., USA) supplemented with 5% fetal calf serum at 37°C in an atmosphere of 5% CO_2 . The cells were activated with 20 U/ml interferon γ (Sigma, USA) and 100 ng/ml lipopolysaccharide (LPS; *E. coli* 0111 B:4; Sigma, USA). After 24 h culture the supernatants were collected and frozen at -80°C until used.

Measurement of cell viability

Viability of the cells was routinely monitored by cellular exclusion of trypan blue. In some experiments, cell respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT, Sigma, USA) to formazan.

Cytokine determination

Cytokine concentrations in culture supernatants were measured using capture ELISA as described previously¹⁷. For interleukin IL-6, IL-10, and IL-12, microtiter plates (Corning, USA) were coated overnight with rat monoclonal antibody (mAb) against a mouse cytokine (capture antibody). After blocking the plates with 4% albumin (2 h), standards and tested supernatants were added and incubated overnight. Finally, the plates were coated with biotinylated antibodies against the same cytokine-detecting antibody for 1 h. The ELISA was developed with HRP streptavidin (Vector, USA) followed by OPD and H_2O_2 (both Sigma, Germany) as substrates for 30 min. For TNF- α , a peroxidase-conjugated goat anti-rabbit IgG (Sigma, USA) was used to develop the reaction. The reaction was stopped with 3 M H_2SO_4 .

and the optical density of each well was measured at 492 nm in a 96-well plate reader. Tween 20 (0.1%) in phosphate buffer was used as a washing solution. The following reagents were used for the following assays:

- IL-6 – rat anti-IL-6 and biotinylated rat anti-IL-6 (both Pharmingen, USA) mAbs were used as detecting antibodies. Recombinant mouse IL-6 (PeproTech, USA) was used as a standard. The detection limit was about 15 pg IL-6/ml.
- IL-10 – rat anti-mouse IL-10 and biotinylated rat anti-mouse IL-10 (Pharmingen, USA) mAbs were used as detecting antibodies. Recombinant mouse IL-10 (Pharmingen, USA) was used as a standard. The limit of detection was 15 pg IL-10/ml.
- IL-12 p40 – biotinylated rat anti-mouse IL-12 (p40) mAbs, clone 17.8 (Pharmingen, USA) and biotinylated rat anti-mouse IL-12 (Endogen, USA) were used as capture and detecting antibodies. Recombinant mouse IL-12 (Genzyme, UK) was used as a standard. The detection limit was about 30 pg IL-12 p40/ml.
- TNF- α – rat anti-murine TNF- α and biotinylated rat anti-mouse TNF- α (Pharmingen, USA) mAbs were used as detecting antibodies. Recombinant mouse TNF- α (Pharmingen, USA) was used as a standard. The limit of detection was 30 pg TNF- α /ml.

Nitrite determination

Nitric oxide (NO), quantified by the accumulation of nitrite as a stable end product, was determined by a microplate assay¹⁰. Briefly, 100 μ l samples (supernatants of macrophages) were removed from supernatants and incubated with an equal volume of Griess reagent (1% sulphanilamide 0.1%, *N*-1-naphthylendiamine, dihydrochloride 2.5% H_3PO_4 ; Sigma, USA) at room temperature for 10 min. The absorbance at 550 nm was measured with a microplate reader. Nitrite concentration was calculated from a sodium nitrite standard curve.

Statistical analysis

The statistical significance of differences between groups was analyzed using a factorial ANOVA (Microsoft Excel) followed by the Student's *t*-test, if appropriate. Results are expressed as mean $\pm$ SE. The differences were regarded as significant for $p < 0.05$.

RESULTS

Pathogenic capacity of PA and SE

The pathogenic potentials of PA and SE were evaluated according to their ability to evoke local skin inflammation after intradermal injection of live bac-

Table 1. Inflammatory reaction induced by *Propionibacterium acnes* (PA) and *Staphylococcus epidermidis* (SE)

Bacteria injected	Local inflammatory reaction	
	incidence	severity
PA	7/26 (27%)	0 [19] + [3] ++ [4] +++ [0]
SE	0/26 (0%)	0 [26]

The inflammation was evoked by intradermal injection of 10^8 live bacteria into the left ears of mice. As a control, a 0.9% NaCl solution was injected intradermally into the right ears. The mice were infected either with PA or SE. Observations were carried out for 3–4 weeks (for details see Materials and Methods). The evaluation scale of severity of local inflammation consisted of 4 grades: 1) 0: no lesions; 2) +: ear thicker than the reference ear; 3) ++: ear thicker and reddened; 4) +++: purulent lesions within the ear, erosion; [19] – number of mice which expressed the inflammatory reaction of the indicated severity.

teria into the ear lobe. Inflammation was characterized by a doubling of ear thickness at 72 h and a peak of more than 3 times normal thickness on days 14–21. As shown in Table 1, PA evoked a local inflammatory reaction of low intensity in 7 out of 26 mice studied (incidence 27%). This reaction was mild and transient. In this group of mice neither general symptoms were found nor did a single animal die. SE caused no lesions in the ears of the animals studied.

The adjuvant properties of PA and SE

In order to determine adjuvant properties of PA and SE, mice were immunized with protein antigen (OVA) alone or together with the tested bacteria. It was found that PA emulsified with OVA stimulated the production of IgG anti-OVA antibody 5 times more efficiently than OVA alone or OVA with SE (no adjuvant effect; Fig. 1).

The effects of PA and SE on the production of ROS and NO by macrophages

The ability of the tested bacteria to induce the production of reactive oxygen species (ROS) by macrophages *in vitro* was evaluated using luminol-dependent chemiluminescence (LCL). LCL observed during the respiratory burst of phagocytes is commonly attributed to the production of O_2^- and H_2O_2 ². As shown in Fig. 2, macrophages released similar amounts of ROS in response to both bacterial strains.

We also tested the influence of the bacteria on the generation of NO by peritoneal macrophages. No sig-

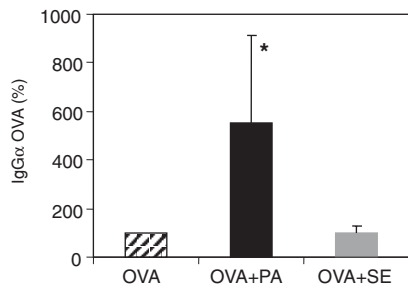


Figure 1. Mice were immunized on day 0 followed by a booster injection (day 21) either with: 3 mg/mouse ovalbumin (OVA) alone (controls), 3 mg OVA mixed with 5×10^8 heat-killed PA (OVA+PA), or OVA mixed with SE (OVA+SE). Mice (8 mice per group) were bled 14 days after the booster and the sera were analyzed by ELISA to measure IgG antibodies specific to OVA (for details see Materials and Methods). Results are expressed in percentage and were calculated as: (the level of serum IgG anti-OVA determined in the experimental groups)/(the level of IgG anti-OVA in serum taken from control mice) $\times 100\%$. Results represent data taken from 3 independent experiments. * $p < 0.05$ (OVA+PA) vs. OVA.

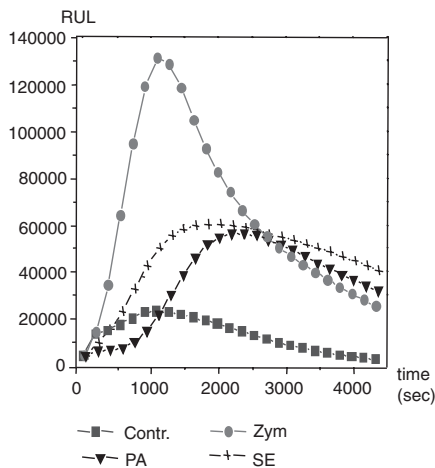


Figure 2. Zymosan-elicited peritoneal macrophages were activated *in vitro* with either PA or SE. As a control, macrophages stimulated with opsonized zymosan (Zym) were used. Luminol-dependent chemiluminescence was performed as described in Materials and Methods. The chemiluminescence signal is expressed as relative light units (RUL) per second. The figure shows the results of 1 of 4 separate experiments.

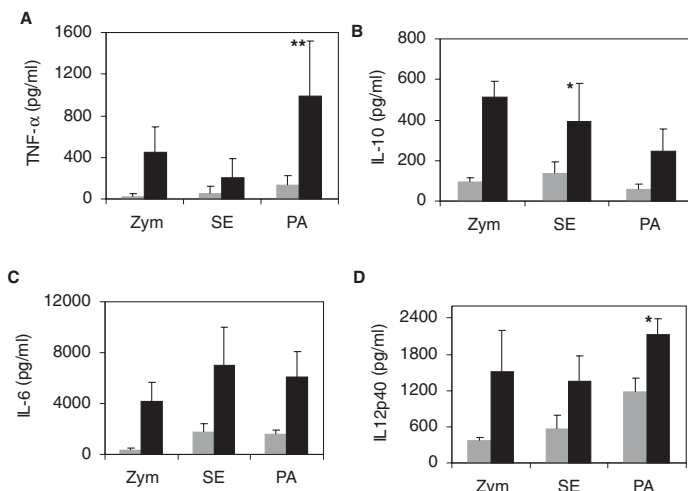


Figure 3. Peritoneal macrophages elicited either with zymosan (Zym), PA or SE were cultured with (■) or without (□) LPS (see Materials and Methods). After 24 h, the supernatants were collected and tested for TNF- α (A), IL-10 (B), IL-6 (C), and IL-12 (D). The results are the mean $\pm$ SE of 5 separate experiments. * $p < 0.05$; ** $p < 0.01$ PA vs. SE.

nificant statistical differences in the level of nitrite, the stable product of NO, were observed between PA ($14.8 \pm 2.2 \mu\text{M}$) and SE ($14.4 \pm 1.8 \mu\text{M}$).

The effects of PA and SE on the release of cytokines by macrophages

Peritoneal macrophages elicited with PA and SE release *in vitro* a distinct pattern of cytokines. As shown in Fig. 3, macrophages released 2 times more TNF- α and IL-12 p40 after incubation with PA than with SE. In contrast, the level of IL-10 was 2.5 times higher in the culture of macrophages elicited with SE than that with PA. The level of IL-6 was similar for both bacteria. Additional *in vitro* stimulation of macrophages with exogenous LPS increased proportionally the amount of all the cytokines tested.

DISCUSSION

It is commonly accepted that PA plays a central role in acne pathogenesis^{5, 8, 14}. On the other hand, PA is not pathogenic by normal standards, because no correlation between the number of bacteria and the severity and type of acne has been found. Nevertheless, this bacterium produces a variety of extracellular products, such as lipases, proteases, and chemotactic factors, which are responsible for the initiation and maintenance of the inflammatory response^{12, 24}. Thus, PA directly contributes to the development of acne via its effects on humoral and cell-mediated immunity, complement activation, and pro-inflammatory mediator production by phagocytic cells^{1, 3, 25}. It has been reported that specific antibodies against PA are involved in the pathogenesis of acne. Ingestion of PA by neutrophils in the presence of antibodies specific to PA results in the release of hydrolases that may contribute to the disruption of the follicular wall⁸.

On the other hand, acne lesions are colonized by two genera of Gram-positive bacteria, *Propionibacterium* and *Staphylococci*²³. Although SE does not seem to be involved in the pathogenesis of acne, there is no clear evidence concerning the relative contributions of these two major skin bacteria to the activation of immune inflammatory cells at the sites of acne lesions.

In order to develop an animal model of inflammation that would be relevant to acne vulgaris, killed PA bacteria are injected intradermally into the ears of rats²⁶. In our model, we injected the ears of mice with live PA or SE to stimulate a local, chronic inflammation. The main difference between the development of acne lesions in humans and the development of skin lesions in the inflamed mice ears is that these animal models essentially bypass the gradual formation of comedones and they develop changes partially equivalent to the stage of comedo rupture. PA (bacteria isolated from acne lesions) induced local inflammation with an incidence of 27%, while SE failed to produce any inflammatory response. Keeping in mind all the restrictions concerning the relevance of animal models to human acne, our results confirmed that PA, but not SE, is responsible for the generation of the skin inflammation in acne vulgaris.

The accumulation and persistent activation of neutrophils and macrophages at the site of inflammation are a hallmark of chronic inflammatory diseases⁷. Recognition of microbial pathogens by the cells of the immune system triggers host defense mechanisms to combat infection. However, activation of these same mechanisms can also result in tissue injury. In acne vulgaris, the host response to PA results in the persistent production of pro-inflammatory cytokines and contributes to the development of acne lesions⁷. Recent studies have demonstrated that the peptidoglycan of PA triggers cytokine responses via Toll-like receptor 2 (TLR2). Since the peptidoglycan of PA is distinct from that of most Gram-positive bacteria (e.g. SE), the effect of PA on cytokine production by TLR2 cells may be unique, promoting tissue injury by these cells. Moreover, the detection of TLR2 cells in the perifollicular region provides indirect evidence that TLR2 activation contributes to the pathogenesis of acne¹⁵.

Given these data, PA, but not SE, along with TLR2 are logical targets for therapeutic intervention to block the detrimental inflammatory response in acne. Only recently have we found that taurine bromamine (TauBr), at non-cytotoxic concentrations, selectively killed PA, though not affecting SE (manuscript in

preparation). Moreover, TauBr, similarly to taurine chloramine, shows anti-inflammatory capacity by inhibiting the generation of pro-inflammatory cytokines and NO by phagocytic cells^{16, 18, 20}. Thus the topical application of bactericidal agents selective for PA that also exert anti-inflammatory properties seems to be a promising new strategy in the topical treatment of patients with acne vulgaris.

In this study we have shown that the peritoneal exudate cells (mostly macrophages) elicited by PA and SE differ in their profiles of secreted cytokines. PA elicited macrophages which preferentially generate TNF- α and IL-12, the cytokines that have pro-inflammatory properties and mediate Th1-type immune response. In contrast, the peritoneal macrophages elicited by SE preferentially release IL-10, an anti-inflammatory cytokine. Interestingly, activation of these cells with LPS *in vitro* does not change the balance between pro- and anti-inflammatory cytokines, which confirms that PA and SE differentially affect the function of inflammatory cells. In addition, our present data concerning the adjuvant properties of the bacteria tested clearly indicate that PA, but not SE, may enhance antibody response to heterogeneous antigen. These results are in agreement with previous reports⁶. The differences in adjuvant properties between PA and SE may be explained by their differing abilities to induce cytokine production by activated macrophages (potential antigen-presenting cells), as described above.

Except for pro-inflammatory cytokines, a variety of agents generated by activated phagocytic cells, such as ROS and NO, may play a role in mediating acne lesions^{1, 11, 13, 21}. For example, the increased ability of neutrophils to produce ROS in patients with acne reveals their involvement in neutrophil-dependent tissue damage¹. Moreover, antibiotics used for the treatment of acne significantly inhibited the generation of ROS by neutrophils compared with other antibiotics¹. In order to determine the ability of PA and SE to stimulate phagocytic cells for the generation of bactericidal agents, the generation of ROS and of NO was investigated. We found that both bacteria are equally potent as stimulators of the generation of ROS and NO *in vitro*, while, *in vivo* the contributions of PA and SA to neutrophil attraction and activation seem to be different^{1, 7}.

These discrepancies may be explained by the biofilm concept of chronic infections. This new microbial concept assumes that the formation of a biofilm in tissues plays a key role in the cross-talk between bacteria and host defense system and is a common cause of persistent infections, including acne vulgaris^{7, 9}.

Indeed, the biofilm PA bacteria differ from their planctonic counterparts by resistance to phagocytic cells and antibiotics⁷.

In conclusion, differences between PA and SE in their ability to induce local skin inflammatory response and stimulate the production of pro-inflammatory mediators by neutrophils and in their adjuvant properties indicate different roles of PA and SE in the pathogenesis of acne vulgaris. These

observations are in agreement the studies of Ashbee et al.³ They have shown that while the antibody response to SE is relatively harmless, antibodies to PA may be involved in the pathogenesis of acne. Thus it is unlikely that SE, in contrast to PA, contributes to skin lesions in acne. On the other hand, SE, but not PA, is a common skin pathogen in chronic medical device-associated infections due to its ability to form multilayer biofilms on polymeric surfaces⁹.

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