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Antibodies Elicited by Inactivated *Propionibacterium acnes*-Based Vaccines Exert Protective Immunity and Attenuate the IL-8 Production in Human Sebocytes: Relevance to Therapy for Acne Vulgaris

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Abstract

Propionibacterium acnes is a key pathogen involved in the progression of inflammation in acne vulgaris. We examined whether vaccination against *P. acnes* suppressed *P. acnes*-induced skin inflammation. Inactivation of *P. acnes* with heat was employed to create a *P. acnes*-based vaccine. Intranasal immunization in mice with this inactivated vaccine provoked specific antibodies against *P. acnes*. Most notably, immunization with inactivated vaccines generated *in vivo* protective immunity against *P. acnes* challenge and facilitated the resolution of ear inflammation in mice. In addition, antibodies elicited by inactivated vaccines effectively neutralized the cytotoxicity of *P. acnes* and attenuated the production of proinflammatory cytokine IL-8 in human sebocyte SZ95 cells. Intranasal immunization using heat-inactivated *P. acnes*-based vaccines provided a simple modality to develop acne vaccines. These observations highlight the concept that development of vaccines targeting microbial products may represent an alternative strategy to conventional antibiotic therapy.

INTRODUCTION

Propionibacterium acnes, a gram-positive anaerobic bacterium, is a member of the resident bacterial flora and mostly resides in pilosebaceous follicles. It has been known that the bacterium plays a critical role in the development of inflammatory acne vulgaris, which is the most common disease of human skin afflicting up to 80% of individuals through their lives (Cunliffe and Gollnick, 2001b; Leyden, 2001; Bojar and Holland, 2004). In inflammatory acne lesions, the follicular epithelium is damaged and a dermal inflammation occurs. So far, several mechanisms of developing acne associated with *P. acnes* have been proposed. The mechanism for the onset of acne can generally be divided into two stages. The first stage is comedo formation, which is characterized by the initiation of inflammatory events before hyperproliferation of the follicular epithelia (microcomedo) (Jeremy *et al*.,

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2003); the second stage is the occurrence of inflammation and disruption of follicular epithelia. Although the involvement of *P. acnes* in the initiation of comedogenesis is still controversial (Cunliffe and Gollnick, 2001a), they can aggravate or intensify abnormal desquamation once overgrowth and colonization of *P. acnes* in the microcomedo occur (Leeming *et al*., 1988; William J Cunliffe, 2001). *P. acnes* produces a number of extracellular enzymes and metabolites that can directly damage host tissues (Hoeffler, 1977; Holland *et al*., 1981; Cove *et al*., 1983; Hoffler *et al*., 1985). In addition, it was also widely accepted that inflammation in acne lesions may be induced by host immune reactions to *P. acnes*. Chemotactive substances released from the bacteria attract polymorphonuclear leukocytes (Webster and Leyden, 1980). It has also been reported that *P. acnes* stimulates production of proinflammatory cytokines, including IL-1β, IL-8, IL-12, and tumor necrosis factor-α. The *P. acnes*-induced cytokine productions have known to be mediated by toll-like receptor (TLR) 2 (Kim *et al*., 2002; Kim, 2005; Nagy *et al*., 2006).

Reduction in *P. acnes* numbers by antimicrobial agents correlates with clinical improvement of acne in patients, therefore antibiotic therapy has been a mainstay of treatment for acne over the past 25 years. However, *P. acnes* strains with clinically significant antibiotic resistance, and multiple drug resistance were identified from acne patients with long-term antibiotic treatment (Eady *et al*., 2003; Nord and Oprica, 2006). More recently, it has been demonstrated that biofilm formation by *P. acnes* increases resistance against antimicrobial agents (Coenye *et al*., 2007). In addition, *P. acnes* resists killing by phagocytes and is able to survive in macrophages (Webster *et al*., 1985b). These problems may be the roots of clinical failure to treat acne. In the cases of mild and severe acne, acne lesions are observed not only on the face, but also on the chest, shoulders, or back of the patient. In such cases, topical treatment with anti-acne agents in large areas or for long periods of time may produce side effects, and systemic antibiotic therapy may nonspecifically kill skin bacteria, which impacts the homeostasis of skin-resident microflora (Ochsendorf, 2006). Isotretinoin, 13-*cis*-retinoic acid, is a vitamin A-derived retinoid that has been widely prescribed for systemic treatment of severe acne. This agent acts primarily by decreasing the size of the sebaceous glands and reducing sebum production by up to 90%, thereby inhibiting the growth of *P. acnes* and subsequent inflammation (Clarke *et al*., 2007). However, its use has now been highly regulated because of its adverse effects, and it is not appropriate for most acne patients. Currently available treatments for acne lesions are often only palliative and minimally effective while treatment is maintained. In most cases, when treatments are discontinued, recurrence of acne often results. Therefore, acne is still one of the skin diseases that require alternative approach of treatments, although various antibiotics, medicines, and therapies for acne have been developed.

It has been demonstrated that IgG-coated bacteria were found in comedones of acne patients, suggesting that IgG in comedones was derived from the serum and selectively accumulated in the follicle (Knop *et al*., 1983). This observation inspired us to hypothesize that suppression of *P. acnes* by anti-*P. acnes* antibody may have a potential to prevent its progression and pathogenicity. In this study, we demonstrated protective effects of vaccination with heat-killed *P. acnes* on *P. acnes*-induced inflammation. More importantly, the model employing the production of proinflammatory cytokines from human sebocytes provides a relevance to the vaccine therapy for acne vulgaris. Furthermore, inactivated *P. acnes*-based vaccines afford a straight-forward modality for vaccine manufacturing.

RESULTS

P. acnes-induced inflammation in mice

To induce a model of *P. acnes* inflammation, we injected 10^7 colony-forming units (CFU) of living *P. acnes* intradermally into mouse ears. Significant cutaneous erythema (Figure 1a,

left ear), ear swelling, and granulomatous response (Figure 1b) were observed in *P. acnes*-injected ear 24 hours after the bacterial injection, but neither of them were induced by phosphate-buffered saline (PBS) injection (Figure 1a, right ear, and c). Histological observation revealed that injection of *P. acnes* induced a considerable increase in the number of infiltrated inflammatory cells (Figure 1b).

Immunogenicity of heat-killed *P. acnes*

To generate sufficient and specific antibody against *P. acnes*, mice were intranasally immunized with heat-killed *P. acnes* three times at a 3-week interval. Serum was collected 1 week after the third inoculation. Data from western blot indicated that two main components (approximately 64 and 250 kDa) of *P. acnes* were immunoreactive to antibodies elicited by heat-killed inactivated *P. acnes* (Figure 2, lane 1). No immunoreactivity to *P. acnes* lysates was detected if serum from PBS-injected mice was used (Figure 2, lane 3). In addition, antibodies in the sera from *P. acnes*-immunized mice did not cross-react with proteins in *Staphylococcus epidermidis* (ATCC 12228) (Figure 2, lane 2), indicating the specificity of anti-*P. acnes* antiserum. Intranasal administration also demonstrated the competence of inactivated *P. acnes*-based vaccines in inducing mucosal immunity.

In vivo protective effect of inactivated *P. acnes*-based vaccines

To determine the protective immunity elicited by vaccination with heat-killed *P. acnes*, the ear of the vaccinated mouse was intradermally challenged with living *P. acnes* (1×10^7 CFU). The increase in *P. acnes*-induced ear thickness was recorded until thickness subsided (Figure 3). A biphasic pattern of changes in ear thickness was observed. In the PBS-inoculated mice, ear thickness increased rapidly more than two-fold (215.8%) on the first day, decreased on the second day, then rebounded (225.0%) on the seventh day, and recovered 78 days after bacterial challenge. The increase in ear thickness in both phases ($P = 0.04$ at 24 hours and $P = 0.0013$ on 7 days post-challenge) was significantly suppressed when mice were immunized with inactivated *P. acnes*-based vaccines. In *P. acnes*-immunized mice, the increase in ear thickness completely subsided 22 days after bacterial challenge. These data strongly demonstrated that vaccination with inactivated *P. acnes*-based vaccines suppressed the bacterial progression and facilitated the recovery of *P. acnes*-induced inflammation.

In vitro neutralization of *P. acnes*-induced proinflammatory cytokine production in human sebocytes

A human SZ-95 sebocyte was utilized with the aim of establishing a model for evaluating the potency of anti-*P. acnes* antiserum to neutralize the cytotoxicity of *P. acnes*. It has been shown that activation of TLR2 expressed in human sebocytes with *P. acnes* notably increased cytokine IL-8 production (Nagy *et al*., 2006). In addition, elevation of IL-8 and its gene expression was observed in skin biopsies of patients with inflammatory acne vulgaris (Trivedi *et al*., 2006; Abd El All *et al*., 2007). Thus, the capability of anti-*P. acnes* antiserum to neutralize *P. acnes*-induced IL-8 production in human sebocytes was examined. Sebocytes produced 1.2 ng ml^{-1} of IL-8 when they were treated with the bacteria that were pre-incubated with serum from PBS-inoculated mice (Figure 4a). By contrast, pre-incubation with anti-*P. acnes* antiserum effectively decreased *P. acnes*-induced IL-8 production to 0.59 ng ml^{-1} ($P = 0.0015$). Pre-incubation of 2 hours with antisera did not influence the growth of *P. acnes* as determined by CFU (Figure S2). These results suggest that anti-*P. acnes* antiserum attenuated *P. acnes*-induced IL-8 production in sebocytes without affecting bacterial survival. The ability of anti-*P. acnes* antiserum to neutralize the cytotoxicity of *P. acnes* was also determined. After incubation with serum from PBS-inoculated mice, *P. acnes* triggered 29.3% of sebocyte death (Figure 4b). On the other hand, the dead sebocytes were dramatically reduced to 12.7% ($P = 0.003$) when cells were treated

with neutralized *P. acnes*. These data indicated that antibodies evoked by inactivated *P. acnes*-based vaccines efficiently counteracted the *P. acnes*-induced inflammation.

DISCUSSION

In acne lesions, a partially occluded follicle creates an ideal anaerobic environment for *P. acnes* to multiply. Consequently, the increase of *P. acnes* and its enzymes, virulence factors, and pattern recognition ligands stimulated the skin resulting in inflammation and acne lesions (Leyden, 2001; William J Cunliffe, 2001; Bojar and Holland, 2004). Our results indicated that injection of *P. acnes* into ICR mouse ears induced an increase in the ear thickness (Figure 1) and granulomatous response (Figure 1b). One day after injection, we observed that *P. acnes* was surrounded by a densely packed granulomatous infiltrate. Although ears injected with *S. epidermidis* (ATCC 12228; 10^8 CFU), an aerobic skin commensal, induced a minor swelling, this swelling rapidly subsided within 4 days (data not shown). Although multiple injections of *P. acnes* into mouse ears may cause tissue necrosis, it is worth investigating whether TLR2 tolerance induced by a repeat of intraperitoneal injection of *P. acnes* could alter host sensitivity to bacterial infection (Medvedev *et al*., 2006). Mice produce little or no triglycerides, a fact that has hindered the development of an animal model for studying the lipogenesis in acne lesions (Webster *et al*., 1981b). The rabbit ear model has a lack of bacterial colonization and inflammation (Mirshahpanah and Maibach, 2007). In addition, the use of rabbits may be inconvenient for vast vaccinations. Rhino mice with utricles that create larger follicle size have been widely used for screening anti-acne drugs (Takaoki and Kawaji, 1980). However, as Rhino mice with deficits in immune system cannot produce antibodies against thymus-dependent antigens (Takaoki and Kawaji, 1980), we thus created inflammatory responses in an ICR mouse strain as an animal model (De Young *et al*., 1984, 1985) to evaluate the potency of inactivated *P. acnes*-based vaccines. In most cases of acne, *P. acnes* is thought to colonize on the skin surface and/or within sebaceous follicles. Bacteria could enter the dermis once the follicular wall was ruptured (Kligman, 1974). Injection of *P. acnes* into mouse ears may represent an animal model for the granulomatous type of acne inflammation that follows follicular rupture.

Many vaccine development approaches are under investigation, but the one straightforward method is the use of intranasally administered killed whole pathogen preparations. It has known that *P. acnes* itself is a potent immunomodulator (Mussalem *et al*., 2006). Our data illustrated that mice immunized with heat-killed *P. acnes* produced antibodies against two *P. acnes*-specific proteins with molecular weights at approximately 64 and 250 kDa. Antibodies against *P. acnes* can be frequently found in severe acne patients (Webster *et al*., 1985a). In some cases, there is a correlation between the severity of acnes and titers of antibodies against *P. acnes* in patients' sera (Holland *et al*., 1986; Ingham *et al*., 1987; Ashbee *et al*., 1997). The antibodies are generated against *P. acnes* exocellular enzymes, or cell wall/membrane fractions, such as polysaccharide, carbohydrate, or membrane-binding proteins in acne patients (Dalen *et al*., 1980; Iversen *et al*., 1985; Webster *et al*., 1985a; Ingham *et al*., 1987; Lodes *et al*., 2006). However, acne lesions recurred often, despite antibodies against *P. acnes* having been generated in those patients. One possible explanation could be that those acne patients may not produce sufficient protective antibodies against key virulence factors of *P. acnes* for the suppression of bacterial progression and the prevention of recurrence. Additionally, it is worth examining whether acne patients produced antibodies against the two antigens (approximately 64 and 250 kDa) that were elicited in the mice vaccinated with heat-killed *P. acnes* (Figure 2). As mucosal vaccination with most inactivated organisms induced a relatively weak immune response, adjuvant such as *Vibrio cholerae* cholera toxin or *E. coli* heat-labile enterotoxin was frequently administrated along with antigen to nasal cavity. However, these adjuvants may cause adverse effects (Spangler, 1992). The high immunogenicity of heat-killed *P. acnes*

without adjuvants suggested that inactivated *P. acnes* exerted a self-adjuvant effect. Most importantly, the use of inactivated *P. acnes*-based vaccines eliminates the time-consuming steps required for antigen purification. Furthermore, intranasal immunization circumvents the intrinsic problems associated with multiple needle injections. Thus, *P. acnes*-based vaccines are beneficial for large-scale and rapid vaccine production.

The vaccination effectively suppressed *P. acnes*-induced ear swelling and expedited the recovery of ear inflammation (Figure 3). Ear thickness was measured regularly for 78 days, revealing a biphasic ear-swelling pattern. This is consistent with previous findings illustrating a biphasic change in the activity of the mouse reticuloendothelial system after intraperitoneal injection with phenol-treated *P. acnes* (Kobayashi *et al*., 1980). The biphasic pattern was interpreted by the fluctuation in the number of macrophages and other host cells in the inflamed skins. We also vaccinated ICR mice with heat-killed *P. acnes* (1×10^8 CFU) one day after intradermally challenging with living *P. acnes* (1×10^7 CFU) (Figure S1). Although ear swelling was suppressed during vaccination, a substantial increase in ear thickness still existed 22 days post-bacterial challenge. The result was different with that of mice vaccinated with heat-killed *P. acnes* for 10 weeks (Figure 3) before bacterial challenge. Ear swelling in mice vaccinated for 10 weeks was rapidly subsided 22 days after bacterial challenge. One possible explanation for this distinction is that vaccination after bacterial challenge may not evoke sufficient antibodies to effectively combat bacteria. It has been documented that inflammation in acne lesions was initiated by the production of proinflammatory cytokines after interaction of *P. acnes* to TLR2 (Kim *et al*., 2002;Kim, 2005;Nagy *et al*., 2006). In addition, it has been known that activation of TLR2 expressed in human sebocytes, one of target cells of *P. acnes*, with *P. acnes* released tremendous cytokine IL-8 (Nagy *et al*., 2006;Abd El All *et al*., 2007). The prevalence of *P. acnes* in the sebocytes of sebaceous glands is highly associated with acne progression. Sebocytes were involved in skin immunity by producing antimicrobial substances and proinflammatory cytokines (Nagy *et al*., 2006). Our data revealed that anti-*P. acnes* antiserum effectively neutralized the cytotoxicity of *P. acnes* and lowered *P. acnes*-induced IL-8 production in human sebocytes. The peptideglycan–polysaccharide complexes and lipoteichoic acids, two major cell wall components in the gram-positive bacteria, can stimulate the release of proinflammatory cytokines from monocytes (Mattsson *et al*., 1993). Webster *et al*. (1985a) demonstrated that a carbohydrate-structured component of *P. acnes* was highly antigenic in severe acne patients. Moreover, Basal *et al*. (2004) found a major 96 kDa antigenic component, which stimulated the production of IL-8 and tumor necrosis factor-α from human peripheral blood mononuclear cells. Taken together, inactivated *P. acnes*-based vaccines may diminish inflammation via blocking the TLR2 signaling in sebocytes. Ingham *et al*. (1987) detected antibodies against *P. acnes* exocellular enzymes, that is, lipase and hyaluronate lyase, in normal subjects and acne patients. They, however, could not find any differences of the antibody titers between these two groups, suggesting that the amounts of antigens secreted from *P. acnes* existing in the sebaceous follicles of acne patient may not be enough for humans to elevate antibody titers. Thus, boosting sufficient antibodies against *P. acnes* may be a future direction to develop therapeutic acne vaccines.

It has been reported that mice administrated with *P. acnes* enhanced the resistance to influenza virus (Gangemi *et al*., 1983) and tumor formation (Murano and Cummins, 1989; Mussalem *et al*., 2006) as well as augmented phagocytic activities (Webster *et al*., 1979, 1981a; Gangemi *et al*., 1983). These data implied that inactivated *P. acnes* vaccines may increase host immunity. Adversely, vaccination targeting whole bacterium may lack specificity (Kobayashi *et al*., 1980). Genomic (Bruggemann *et al*., 2004; Bruggemann, 2005) and/or proteomics approached may help in antigen selection for the development of component acne vaccines. We recently selected a surface sialidase from the *P. acnes* genome as an antigen for the development of a component acne vaccine (Nakatsuji *et al*.,

2008). The bacterial specificities of *P. acnes*- and sialidase-based vaccines will be addressed in the future studies.

Data from bacterial counting on agar plates showed that *P. acnes* did not change its growth after incubation with anti-*P. acnes* antiserum (Figure S2). Additionally, counting bacteria from homogenized ear skins indicated that intranasal vaccination did not statically alter the colonization of *P. acnes* (data not shown). To confirm this phenomenon, we counted *P. acnes* in fluids extracted from an implanted tissue chamber in vaccinated and nonvaccinated mice (Nakatsuji *et al*., 2008). Consistently, vaccination did not change the multiplication of *P. acnes* in mice (Figure S3). These results suggested that intranasal immunization with inactivated *P. acnes*-based vaccines may not produce sufficient bactericidal antibodies. On the other hand, the immunization with an acne vaccine that has no bactericidal action may perform no risk of destroying the balance of skin microflora. Although the killed strains of *P. acnes* and/or Staphlococci have been used as acne vaccines and named as acnevac or autovaccines (Zaluga, 1998), their potency in inhibiting the production of proinflammatory cytokines in human skin cells is undetermined. In conclusion, we demonstrated that vaccination with inactivated *P. acnes* suppressed inflammation *in vitro* and *in vivo*. The fact that antibodies effectively suppressed the production of proinflammatory cytokine IL-8 in human sebocytes highlights inactivated *P. acnes*-based vaccines as a novel treatment for acne vulgaris.

MATERIALS ANS METHODS

Culture of *P. acnes*

Propionibacterium acnes ATCC 6919 (American Type Culture Collection, Manassas, VA) was cultured on Brucella broth agar (BD, Sparks, MD), supplemented with 5% (v/v) defibrinated sheep blood (LAMPIRE Biological Laboratories, Pipersville, PA), vitamin K (5 μgml^{-1}, Remel, Lenexa, KS), and hemin (50 μgml^{-1}, Remel, Lenexa, KS), under an anaerobic condition using Gas-Pak (BD, Sparks, MD) at 37°C. Single colony was inoculated in Reinforced Clostridium Medium (Oxford, Hampshire, England) and cultured at 37°C until reaching OD_{600} = 1.0–3.0 (logarithmic growth phase) under the anaerobic condition. Bacteria were harvested by centrifugation at 5,000 × *g* for 10 minutes, washed with PBS three times, and suspended to appropriate amount of PBS for the experiments.

Intranasal vaccination of mice with heat-killed *P. acnes*

Female 8-week-old ICR mice (Harlan, Indianapolis, IN) were used in all experiments. Mice were housed according to institutional guidelines. *P. acnes* was suspended in PBS and inactivated by heating bacteria at 60°C for 30 minutes. After inactivation, *P. acnes* was unable to grow on an agar plate (data not shown). Inactivated *P. acnes* was harvested by centrifuging at 5,000 × *g* for 5 minutes and re-suspended to appropriate amount of PBS. An amount of 25 μl aliquot of inactivated-*P. acnes* suspension (1×10^8 CFU) was intranasally inoculated to an ICR mouse every 3 weeks. The second and third boosts were given at the same manner as the first inoculation. Control mice were inoculated with an equal volume of PBS or *S. epidermidis* (ATCC 12228).

Propionibacterium acnes-induced inflammation

An amount of 20 μl aliquots of living *P. acnes* (1×10^7 CFU) suspended in PBS was intradermally injected in the central portion of the left ear. As a control, 20 μl of PBS was injected into the right ear of the same mice. For histological observation, the ear was cross-sectioned, stained with hematoxylin and eosin (Sigma diagnostics, St Louis, MO), and viewed on a Zeiss Axioskop2 plus microscope (Carl Zeiss, Thornwood, NY). To examine *in vivo* protective effects, ears of immunized mice and their controls were challenged with *P.*

acnes. The increase in ear thickness was measured using a micro caliper (Mitutoyo, Japan) after the bacterial challenge. The increase in ear thickness of *P. acnes*-challenged ear was calculated as % of a PBS-injected control.

Detection of antibody against *P. acnes* by western blotting

After centrifugation, *P. acnes* pellet was suspended in 8 M urea, homogenized by beating with 0.1 mm glass beads for 2 minutes, diluted with sample buffer (125 mM Tris-HCl buffer, pH 6.8, containing 4% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.002% bromophenol blue), and boiled for 2 minutes. The sample (7 μg proteins) was electrophoresed in 10% (w/v) polyacrylamide gel (Bio-Rad, Hercules, CA) and electrophoretically transferred onto poly-vinyliden difluoride membranes (Millipore, Billerica, MA) for 60 minutes at a current of 100 V. The membranes were pre-incubated for 30 minutes in PBS containing 5% (w/v) skim milk, and then incubated with anti-*P. acnes* antiserum or control sera (1:1,000 dilution) at 4°C overnight. Bound antibodies were detected with goat anti-rabbit peroxidase conjugated IgG (1:5,000 dilution, Promega, Madison, WI), and the peroxidase activity was developed with Western lighting chemiluminescence kit (PerkinElmer, Boston, MA).

In vitro neutralization

The immortalized human sebocyte line, SZ95 (Zouboulis *et al*., 1999), was cultured in Sebomed basal medium (Biochrom, Berlin, Germany) supplemented with 5 ng ml^{-1} human recombinant epidermal growth factor (Sigma, St Louis, MO), 10% (v/v) heat-inactivated fetal bovine serum (Mediatech Inc., Herndon, VA), at 37°C under an atmosphere of 5% (v/v) CO_2 in air. *P. acnes* was cultured as described above, washed with PBS by centrifuging, and pre-incubated with 2.5% (v/v) anti-*P. acnes* antiserum or control sera, in which complements were deactivated by heating at 56°C for 30 minutes, at 37°C for 2 hours. For neutralization of IL-8 production, the sebocytes (3×10^6 cells) were co-cultured with 300 μl of the pre-incubation mixtures containing 1.5×10^8 CFU of *P. acnes* and 7.5 μl antiserum for 8 hours. As a background, sebocytes were incubated with antiserum alone. After centrifuging to remove bacteria, the concentrations of IL-8 in the culture medium were determined by ELISA using a Quantikine human IL-8 set (R & D Systems Inc., Minneapolis, MN). *P. acnes*-induced IL-8 production was calculated as the difference of amounts between with and without *P. acnes*. For neutralization of cytotoxicity of *P. acnes*, sebocytes (2×10^5 cells per well) were co-cultured with 100 μl of a pre-incubation mixture containing 2×10^6 CFU of *P. acnes* and 2.5 μl of antiserum for 18 hours. As a control, an equal amount of PBS was added instead of *P. acnes*. As a background, Triton-X was added to get a final concentration of 0.1% (v/v) to lyse sebocytes. After incubation, cell viability of sebocytes was determined with acid phosphatase assay (Martin and Clynes, 1991). Cells were washed with PBS three times and incubated with 100 μl of 10 mM *P*-nitrophenyl phosphate in acid phosphatase assay buffer (1 M sodium acetate buffer, pH 5.5, containing 0.1% (w/v) Triton X-100) for 1 hour at 37°C. Then, 10 μl of 1 N NaOH was added to stop the reaction, and absorbance at 405 nm was measured. Cytotoxicity of neutralizing mixture was calculated as follows: (the OD_{405} difference between without and with *P. acnes* treatment)÷(the OD_{405} difference between without *P. acnes* and with Triton-X treatment) × 100 (%).

Statistical analysis

Data are presented as mean±SE. The Student's *t*-test was used to assess the significance of independent experiments. The criterion $P<0.05$ was used to determine statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CFU	colony-forming units
PBS	phosphate-buffered saline
TLR	Toll-like receptor

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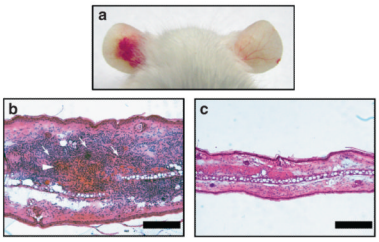

Figure 1. The inflammation in mouse ears after *P. acnes* injection

Ears of ICR mice were injected subcutaneously with 10^7 CFU per 20 μl of *P. acnes* (left ear) or 20 μl of PBS (right ear). (**a**) Inflammation-induced ear redness was visualized 24 hours after injection. (**b, c**) Increase in ear thickness and infiltrated inflammatory cells (arrows) surrounding the injected site of *P. acnes* (arrowhead) were observed in (**b**) a hematoxylin-and-eosin-stained frozen section of *P. acnes*-injected ear. (**c**) Staining PBS-injected ear serves as a control. Bars = 0.2 mm.

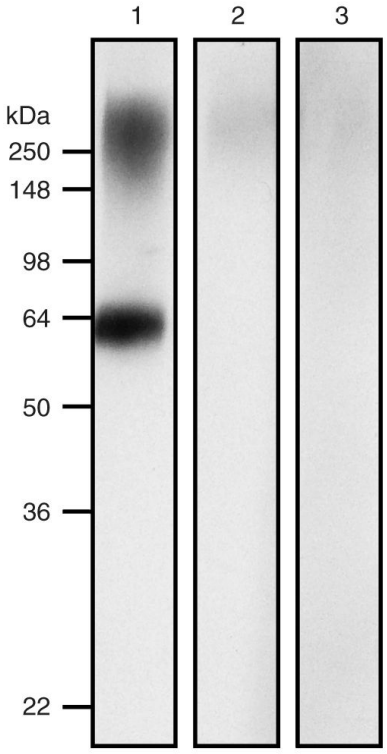

Figure 2. Antibody production after injection with heat-killed *P. acnes*

Lysate of *P. acnes* (7 μg) was separated by SDS-PAGE (10% acrylamide) and then subjected to western blotting using an antiserum obtained from heat-killed *P. acnes* (lane 1), *S. epidermidis*-immunized mice (lane 2), or PBS-injected mice (lane 3) as a primary antibody; immunoreactivity was developed using goat anti-mouse IgG/horseradish peroxidase complex and western lighting chemiluminescence kit (PerkinElmer, Boston, MA). Molecular weights (kDa) are indicated.

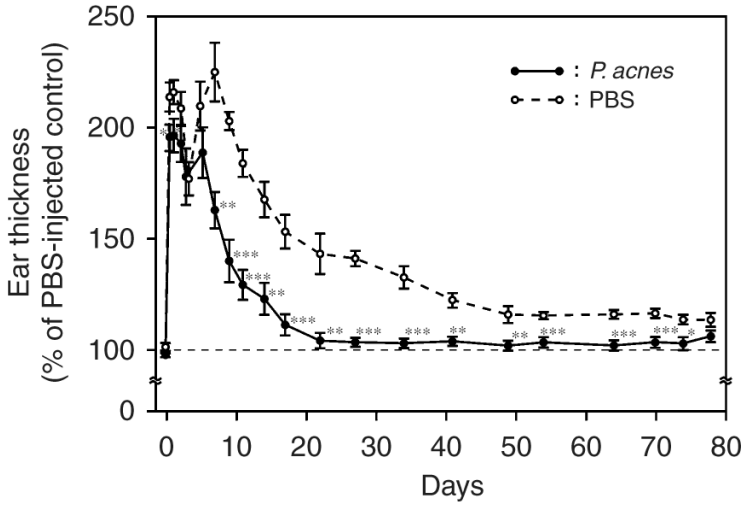

Figure 3. *In vivo* protective immunity in the mice immunized with inactivated *P. acnes*-based vaccines

A total of 20 μl aliquot of living *P. acnes* (1×10^7 CFU) suspended in PBS was intradermally injected into left ears of mice immunized with heat-killed *P. acnes* or PBS (*n* = 8 or 7, respectively). As a control, an equal volume of PBS was injected into the right ears of the same mice. Ear thickness was measured with a micro-caliper at the indicated times post-bacterial challenge. The ear thickness of *P. acnes*-injected ear was calculated as % of a PBS-injected control. Bars represent mean±SE (**P*<0.05, ***P*<0.005, ****P*<0.0005 by Student's *t*-test).

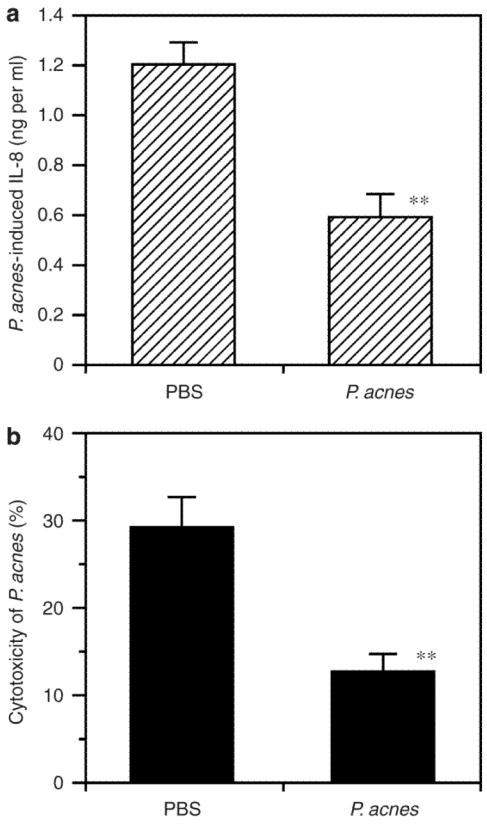

Figure 4. Neutralization of cytotoxicity of *P. acnes* and *P. acnes*-induced IL-8 production in human sebocytes by anti-*P. acnes* antiserum

P. acnes was pre-incubated with anti-*P. acnes* antiserum or anti-PBS control serum (2.5% (v/v)), in which complements were deactivated by heating for 2 hours. (**a**) For neutralization of IL-8 production, the immortalized human sebocytes, SZ-95 (3×10^6 cells), were co-cultured with 300 μl of the pre-incubation mixtures containing 1.5×10^8 CFU of *P. acnes* and 7.5 μl antiserum for 8 hours. Measurement of IL-8 in the culture medium was carried out by ELISA assays. *P. acnes*-induced IL-8 production was calculated as the difference of amount between with and without *P. acnes*.(**b**) For neutralization of cytotoxicity of *P. acnes*, the sebocytes (2×10^5 cells per well) were co-cultured with 100 μl of the neutralization reaction mixtures containing 2×10^6 CFU of *P. acnes* and 2.5 μl antiserum for 18 hours. As a control, an equal amount of PBS was added instead of *P. acnes*. As a background, Triton-X was added to get a final concentration of 0.1% (v/v) to kill sebocytes. After incubation, cell viability of sebocytes was determined with *P*-nitrophenyl phosphate disodium, and cytotoxicity of neutralizing mixture was calculated as follows: (no *P. acnes* group–*P. acnes* added group)÷***(no *P. acnes* group–background group) × 100 (%). Bars represent mean ±SE ($n = 5$). Data were analyzed by Student's *t*-test (**$P<0.005$).