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Intradermal Injection of *Propionibacterium acnes*: A Model of Inflammation Relevant to Acne

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The intradermal injection of 140 μg of *Propionibacterium acnes* (CN 6134) into the ears of female Sprague-Dawley rats produced a chronic inflammation with formation of acneiform lesions. Inflammation was characterized by more than a doubling of ear thickness at 24 h and a peak of 3–4 times control levels at day 21. At 42 days post injection ears were still 3 times normal thickness. Histologically there was early polymorph accumulation giving way to macrophages and lymphocytes by day 7. Pilosebaceous follicles overlying the inflamed area lost their sebaceous glands and became hyperplastic cords of cells that grew down and encapsulated inflammatory loci. By day 9 many of these follicles had become secondary comedones. Three isolates of *P. acnes* from inflammatory acne lesions and 4 of 5 isolates from nonacne patients produced results similar to that of the strain CN 6134. In these cases the number of histologically evident secondary comedones was correlated with ear thickness. In contrast, samples of *Streptococcus lactis, Escherichia coli B*, and *Staphylococcus epidermidis* failed to produce this combination of chronic inflammation and high lesion count. Benzoyl peroxide, tetracycline, erythromycin, phenidone, naproxen, and *cis* and *trans* retinoic acid were inactive as inhibitors of *P. acnes* CN 6134-induced ear thickening. The corticosteroid fluocinolone acetonide produced dramatic suppression of inflammation, but upon cessation of treatment the ears returned to inflamed levels. The specificity for *P. acnes*, the formation of acneiform lesions, and the recalcitrance of the inflammation suggest our model is indeed relevant to acne.

The existing models of acne include rabbit ear comedogenesis [1], the Mexican Hairless dog [2], and human back skin occlusion [3]. Whereas, like acne in humans, all of these models are characterized by the formation of comedones, none approximates the chronic inflammation seen in human inflammatory acne (HIA). This inflammation is the source of discomfort and disfigurement in the acne patient. Current theory on the pathogenesis of inflammatory acne derives from the study of human skin biopsies, and contends that all lesions begin with noninflamed microcomedones, which eventually rupture and release inflammatory stimuli into the adjacent dermis [4]. These inflammatory stimuli consist of sebaceous products, keratinized epithelial cells, and the microflora of the follicle. Of the microflora, much evidence indicates that *Propionibacterium acnes* is the chief culprit [5]. This organism has been intensely studied, not only for its association with acne, but also for its unusual ability to stimulate the reticuloendothelial system (RES) [6].

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Abbreviations:
HIA: human inflammatory acne
RES: reticuloendothelial system

In order to develop an animal model of inflammation that would be relevant to HIA we have intradermally injected the ears of rats with killed *P. acnes*. In essence we have partially mimicked the rupture of a comedone. Here we report that such intradermal injection results in a chronic inflammation, with subsequent encapsulation of inflammatory loci and the formation of acne-like lesions. Furthermore, we show that various strains of *P. acnes* differ in their ability to produce the effect, while the other bacteria tested do not exhibit the effect at all. In addition, the remarkable resistance of the *P. acnes*-induced rat ear inflammation to pharmacologic intervention is demonstrated.

MATERIALS AND METHODS

Rats

Female Sprague-Dawley rats, 100–120 g, from Bantin-Kingman, Fremont, California, were kept under conventional laboratory conditions and used after 1 week of acclimation.

Bacteria Samples

Formalin-killed *P. acnes*, strain CN 6134, was obtained from Burroughs Wellcome, Research Triangle, North Carolina. *P. acnes*, UCLA strains 79, SC, and LAN were isolated from pustular lesions of different patients with moderate inflammatory acne; UCLA strains N1, N2, N3, N4, and 6S were isolated from subjects with normal nonacne skin. All UCLA strains of *P. acnes* were identified by growth characteristics and bacteriophage sensitivity. *Staphylococcus epidermidis* UCLA 1 was isolated from normal human skin. *Streptococcus lactis* UCLA 2 and *Escherichia coli* B UCLA 3 were from the UCLA Department of Microbiology culture collection. All UCLA strains of bacteria were from post log phase cultures grown on brain heart infusion agar and were harvested, heat-killed (95°C, 5 min) and lyophilized, prior to injection.

Injection of Bacteria

Except for the dose-response experiment, all strains of bacteria were diluted to a final concentration of 7 mg/ml in physiologic saline containing 0.01% Thimerosal as a preservative. Saline controls also contained 0.01% Thimerosal. With a 30-gauge needle, bacteria were injected intradermally in 20-μl aliquots (140 μg) in the central, ventral portion of the right ears of ether-anesthetized rats.

Determination of Ear Thickness

Ear thickness was measured using a Peacock Dial Thickness gauge. For each time point, the ears of 5 rats were measured.

Drugs and Treatments

Bacitracin, erythromycin, tetracycline, phenidone, and all-*trans*-retinoic acid were from Sigma Chemical Company, St. Louis, Missouri. *cis*-Retinoic acid (Accutane) was from Hoffmann-La Roche, Nutley, New Jersey. Naproxen and fluocinolone acetonide were from Syntex Research, Palo Alto, California. Benzoyl peroxide was from J. T. Baker Co., Phillipsburgh, New Jersey. Topicycline was from Procter & Gamble, Cincinnati, Ohio. Benzoyl peroxide, phenidone, all-*trans*-retinoic acid, and naproxen were dissolved in acetone. Tetracycline was dissolved in water:acetone (10:90) and bacitracin was dissolved in water:ethanol (10:90). The latter drugs and Topicycline were applied topically to the right ear in a total volume of 40 μl (20 μl to each side of ear). *cis*-Retinoic acid was given by gavage and prepared fresh daily

by resuspending the contents of Accutane capsules in corn oil. This was done in reduced light and the suspension was kept in a light-proof container. For gavage, an appropriate quantity of the drug was delivered in 0.3 ml of corn oil. All drug treatments were begun immediately after *P. acnes* injection. Drug vehicles were used as controls.

Histology

The ears of at least 5 animals were examined at each time point. After animals were sacrificed by CO_2 asphyxiation, the ears were excised and immediately placed in 10% neutral buffered formalin. Following fixation, tissue was dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Sections were viewed under the light microscope.

Lesion Counting

For lesion counting a modification of an existing technique was used [1]. Skin biopsies (4 mm) were punched from the excised ears of sacrificed rats. This diameter included the entire thickened inflamed area. Tissue samples were immediately frozen ventral side down in O.C.T. compound (Lab-Tek Products, Naperville, Illinois) and stored at −70°C for not more than 1 month prior to cryostat sectioning. Sections 10 μm thick were cut in a plane horizontal to the dorsal surface of the ear. Every 4th serially cut section was mounted on a 0.1% poly-L-lysine-coated slide. Sectioning was continued until the cartilage was reached. The tissue was then remounted with the ventral surface up and the sectioning procedure repeated. Sections were stained with hematoxylin and eosin. Lesion counting was performed using the 10× objective of a light microscope. A hyperplastic follicle was scored as a lesion only if it completely lacked sebaceous remnants and contained a central eosinophilic plug of keratin. Such plugs were easily distinguishable from normal hair shafts (see Fig 4). For actual counting, the following sampling technique was used. Each mounted serial section was counted and the number of lesions on the single section with the largest number of lesions on each side of the ear was recorded. Thus the number of lesions per ear was the sum of these two sections.

RESULTS

In a series of preliminary experiments we could find no differences in the effect of formalin vs heat-killed *P. acnes* CN 6134 (results not shown). Since it is commercially available, we used formalin-killed *P. acnes* CN 6134 to standardize our model and will therefore discuss the results obtained with this strain.

Ear Thickness

One day after injection with 140 μg of strain CN 6134, ears were 2–3 times thicker than saline-injected controls at the site of injection. Bacteria-injected ears continued to thicken until day 21 when they were 3–4 times control thickness. At 44 days post injection these ears were still 3 times control thickness (Fig 1). In another experiment ear thickening was found to be dependent on the dose of *P. acnes* injected (Table I).

Histology

Saline-injected ears exhibited only a transient 24-h inflammation at the injection site. In ears injected with strain CN 6134, deposited *P. acnes* could be readily identified as a basophilic mass when animals were sacrificed immediately after injection (Fig 2*A*). At 12 h, a massive infiltration of neutrophils was observed. The cellular infiltrate changed from neutrophils to macrophages and lymphocytes by day 4 (not shown). *P. acnes* injection profoundly changed the appearance of the epidermis and its adnexal structures. By 12 h, inter- and intracellular edema was evident and by 48 h the epidermis was markedly hyperplastic (not shown). The most dramatic changes occurred in the pilosebaceous follicles. The sebaceous glands appeared to undergo a 2- to 3-day period of enlargement followed by their eventual disappearance from many follicles. Follicles overlying the inflamed area became hyperplastic cords of epithelium which grew down to encapsulate the inflamed loci. By 9 days, these encapsulating cords of epithelium had begun to keratinize and secondary comedones containing necrotic inflammatory infiltrate were apparent (Fig 2*B*). When

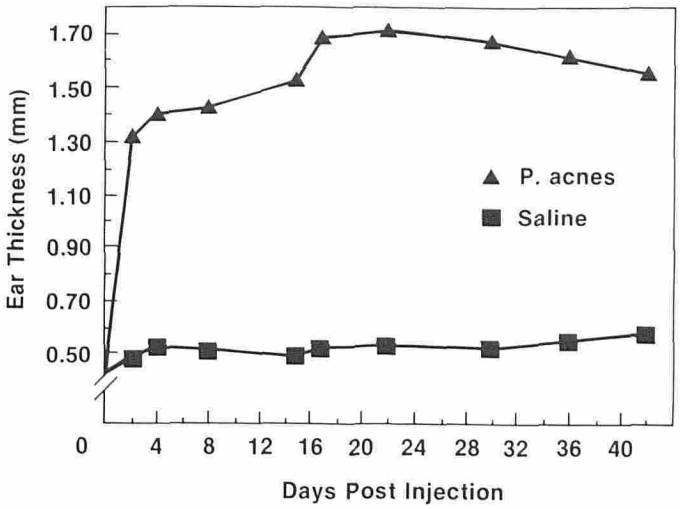

FIG 1. Ear thickening after injection of 140 μg *P. acnes* CN 6134. (▲) *P. acnes*; (■) 20 μl saline. There were 5 animals in each treatment group and time points represent mean measurements. Standard errors were ≤15% of the mean at all time points.

TABLE I. *The effect of different intradermal doses of* P. acnes *(CN 6134) on rat ear thickness*

P. acnes dose (μg)	Δ Thickness (mm × 100)[a] 14 days
140.0	84.2 ± 17.5[b]
35.0	51.1 ± 6.6
17.5	31.4 ± 6.8

[a] Δ Thickness = 14 day thickness − day 0 (preinjection thickness).
[b] Mean ± SE.

sectioned horizontally, similar lesions were seen to be continuous to the surface. These acneiform lesions tended to coalesce with time and eventually could be visualized at the surface as large papule-like structures (Fig 2*C*). These epithelial-lined masses of keratin with inflammatory infiltrate were eventually extruded at the surface leaving crateriform scars behind (Fig 2*D*). Such changes were observed on both the ventral and dorsal sides of injected ears.

The Effect of Drugs on P. acnes*-Induced Ear Thickness*

A variety of drugs, some of which have been reported to have anti-inflammatory effects in human and/or animal skin, were tested for their ability to inhibit strain CN 6134-induced ear thickness. As can be seen in Table II, after 21 daily applications beginning at the time of *P. acnes* injection, none of the tested drugs was effective. Although, as mentioned, some of the drugs in Table II have anti-inflammatory activity in the skin, in no case is dermal inflammation their primary indication for usage. We therefore tested the potent topical steroid, fluocinolone acetonide, for activity against *P. acnes*-induced ear inflammation. In this case, when 10 μg of steroid was applied daily for 8 days beginning at the time of *P. acnes* injection, the increase in ear thickness at 24 h was suppressed by about 70% and the ears gradually returned to noninflamed thickness by day 8 (Fig 3). Remarkably, with the cessation of steroid treatment ears reinflamed, and at day 24 they were actually 2.4 times normal thickness—the same thickness as day 24 inflamed controls (Fig 3).

Lesion Count and Ear Thickness After Injection of Different Bacteria Strains

The histologic appearance of lesions in counted horizontal sections can be seen in Fig 4*B*. It should be emphasized that only follicles containing keratinized plugs were counted. However, many more follicles showed hyperplastic changes. Lesion

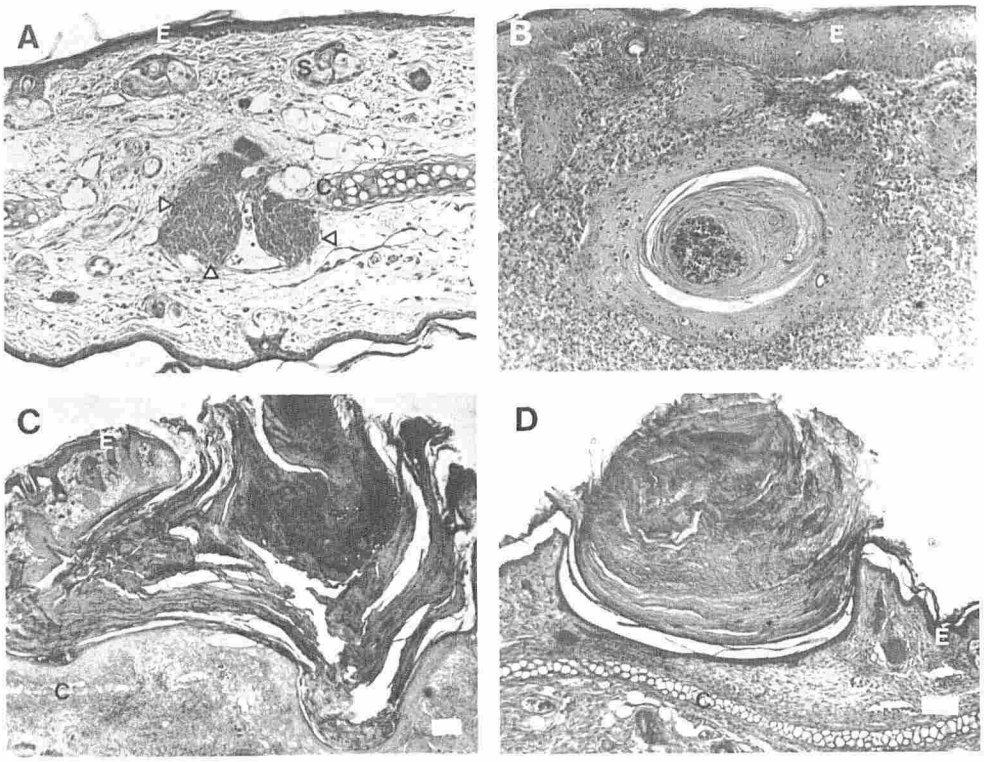

FIG 2. Histology of rat ear after *P. acnes* CN 6134 injection. *A*, Immediately after injection. *Arrowheads* outline area of bacteria deposition. *B*, Nine days. Secondary comedone containing keratin mass and necrotic inflammatory material. Note hyperplastic epidermis and absence of sebaceous glands. *C*, Twenty-nine days. Large acneiform lesion. *D*, Eighty-three days. Keratin plug being extruded at surface. *E* = epidermis; *S* = sebaceous gland; *C* = cartilage. *Scale bars* = 100 μm.

TABLE II. *The effect of various drugs on* P. acnes *(CN 6134)-induced ear thickness*

Drug[a]	Daily dose[b]	Δ Thickness (% control)[c] 21 days
Bacitracin	0.40 mg	107 ± 7
Benzoyl peroxide	4.00 mg	97 ± 11
13-*cis*-retinoic acid	40.00 mg/kg	111 ± 14
Erythromycin	0.40 mg	121 ± 12
Naproxen	1.00 mg	92 ± 13
Phenidone	1.00 mg	130 ± 11
Tetracycline	0.40 mg	121 ± 3
Topicycline	40.00 μl	111 ± 18
Trans-retinoic acid	0.01 mg	128 ± 6

[a] All drugs with the exception of 13-*cis*-retinoic acid (oral route) were topically applied. See *Materials and Methods* for vehicles.

[b] Drugs given daily from day 0 immediately after injection of 140 μg *P. acnes* CN 6134 to day 20.

[c] $\frac{\text{Day 21 thickness} - \text{0 day (preinjection) thickness (drug)}}{\text{Day 21 thickness} - \text{0 day (preinjection) thickness (vehicle control)}} \times 100$. Day 21 control ears were 3–4 times their day 0 thickness.

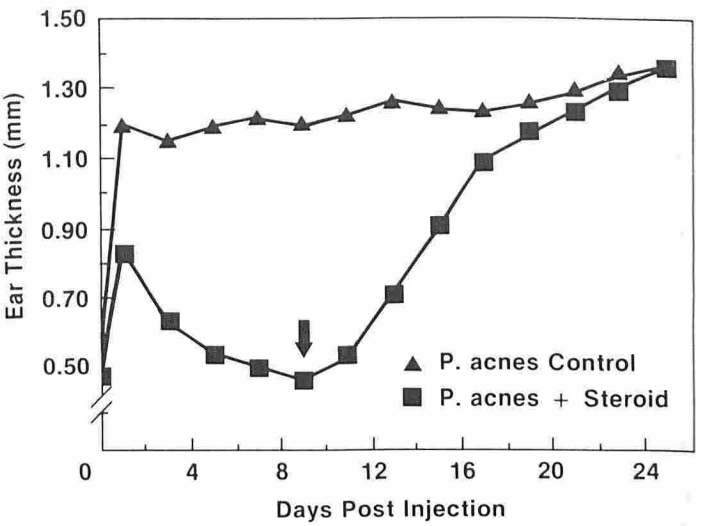

FIG 3. Effect of topical treatment with fluocinolone acetonide. (■) 10 μg fluocinolone acetonide/day. Treatment stopped on day 9 (*arrow*). (▲) 40 μl acetone/day. Treatment stopped on day 9. Both groups of 5 animals each were injected with 140 μg *P. acnes* CN 6134 on day 0. Time points represent mean measurements. Standard errors were ≤15% of the mean at all time points.

counts for various bacteria strains are given in Table III. The ability to produce both an intense ear inflammation as evidenced by the day 7 ear thickness measurements, and a high number of acneiform lesions, as evidenced by the day 15 lesion count, is relatively specific to *P. acnes*. However, not all strains of *P. acnes* were equipotent in this dual ability. For instance *P. acnes* UCLA 79 injection doubled the ear thickness at 7 days and produced a mean lesion count of 7.8 at day 15 while UCLA 6S produced only a 40% thickening and a mean lesion count of 0.2 (Table III). In fact, within the *P. acnes* strains the ability to produce inflammation at 7 days is significantly correlated with the day 15 lesion count as evidenced by linear regression analysis ($r = .83$, $p = .005$). The day 7 thickness was chosen for this analysis because at this time most of the inflammation is due to inflammatory cell infiltrate and edema, while at later times lesions themselves could contribute to ear thickness. It should also be pointed out that although *Streptococcus lactis* injection gave a mean lesion count of 1.0, these lesions were much smaller than those seen in *P. acnes* groups (Table III). Also of interest is the fact that unlike the *P. acnes* groups where moderate inflammation was associated with a moderate lesion count, the moderate inflammation seen in the *Staphylococcus epidermidis*-injected ears was associated with a very low lesion count (Table III).

DISCUSSION

In this communicaton we have described a simple model system of chronic inflammation with relevance to HIA. This relevance is manifested by: (1) the induction of a chronic inflammatory response, (2) involution of the hair follicles and sebaceous glands followed by formation of secondary comedones and eventual scar formation, (3) the specificity of induction of this reaction by *P. acnes*, a microbe long implicated in the mechanism of inflammatory acne, and (4) the chronicity and relative resistance of the inflammation to treatment.

The classical view of inflammatory acne is that each lesion develops from a noninflamed comedone, which eventually ruptures, spills its contents into the dermis, and invokes a chronic

inflammatory response [4]. Our model essentially bypasses the formation of a comedone and begins with a situation partially analogous to comedone rupture. Introduction of large quantities of *P. acnes* into the dermis elicits a massive inflammatory response followed by formation of new acne lesions. This implies that HIA could have a self-perpetuating aspect. Thus, whereas the first lesion could begin with the rupture of a comedone, new inflamed lesions could arise in response to the initial rupture. This idea is supported by the early work of Strauss and Pochi [7], who have shown that injection of human comedonal material into human dermis causes virtually the same sequence of events we see in the rat ear after *P. acnes* injection.

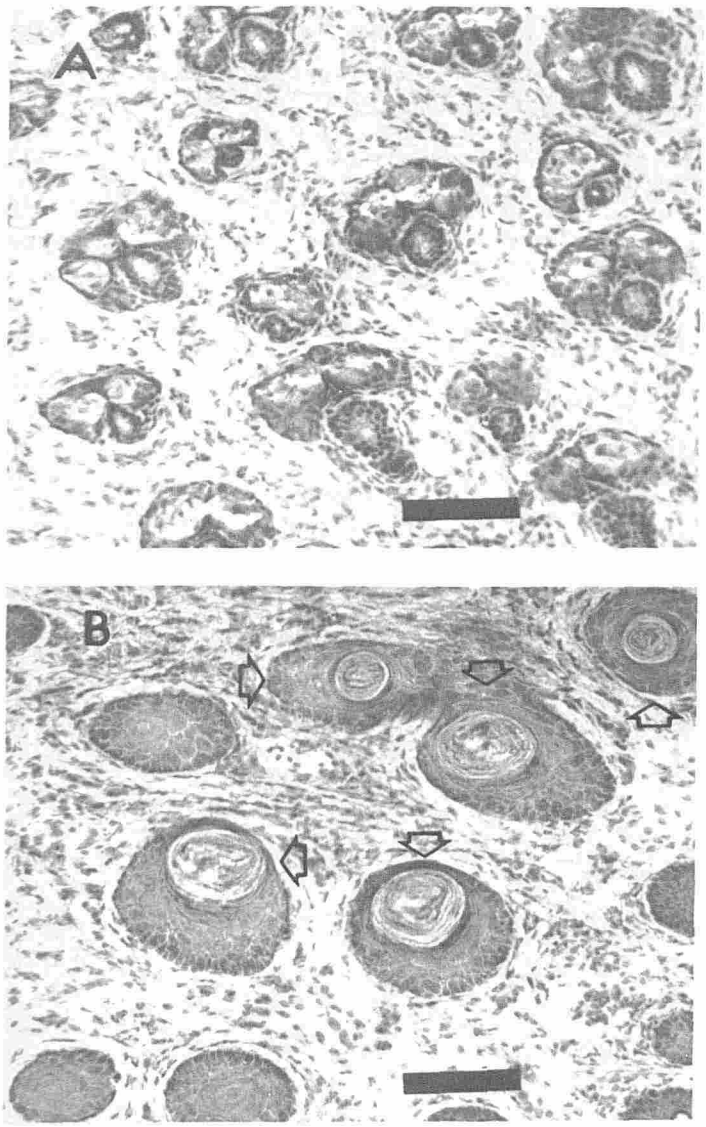

FIG 4. *A*, Horizontal section of day 15 saline-injected control ear showing normal hair follicles and sebaceous glands. *B*, Fifteen days after injection with 140 μg *P. acnes* UCLA 79. *Arrows* indicate scored lesion. *Scale bars* = 100 μm.

The specificity of the rat ear model for *P. acnes* is not surprising. In recent years this bacterium has been shown to have a unique ability to stimulate the RES [6]. A key feature of this ability appears to lie in the resistance of the *P. acnes* cell wall to digestion by macrophages [8]. Intact *P. acnes* can be retrieved from inflammatory cells [8] and organs like the spleen and liver [9] for extraordinary periods after bacteria injection. Significantly, not all strains of *P. acnes* are equipotent in RES-stimulation [6]. Similarly, not all *P. acnes* strains we tested were equipotent in inducing the changes seen in the rat ear. We are currently investigating a possible correlation between RES-stimulating and acneigenic abilities. It is important to emphasize that the work done on the RES utilized killed bacteria. Puhvel and Sakamoto [10] have shown that killed *P. acnes* is equipotent with viable *P. acnes* in ability to elicit an inflammatory reaction in human skin.

Previous reports have shown that tetracycline [11] and 13-*cis*-retinoic acid [12] have cutaneous anti-inflammatory activity. We did not see such an effect in this study. We are currently investigating pre-dosing and alternative routes of administration to determine whether such an effect can be demonstrated.

Leyden [13] has suggested that antibiotics do not produce resolution of existing acne inflammatory nodules, but instead act to inhibit new lesions from occurring. In our opinion, the main challenge of treating existing lesions of HIA is exemplified by the activity in our model of the corticosteroid, fluocinolone acetonide. Here a dramatic suppression of *P. acnes* inflammation was observed for as long as the drug was applied. Cessation of application was followed by a return of ear thickness to inflamed control levels. Apparently, while the steroid could inhibit the inflammatory response to *P. acnes*, the inflammatory stimulus persisted and remained capable of causing intense inflammation. When used in HIA, steroids are generally given intralesionally [14]. At least one author has reported a rebound effect [15], but in general most reports do not follow individual lesions long enough to determine whether this is common.

Of course in HIA, *P. acnes* is probably not the only inflammatory stimulus. Many studies implicate keratin and various lipid products as important inflammatory agents. We are currently investigating these, alone and in combination with *P. acnes*, for their ability to produce chronic inflammation and secondary comedogenesis in the rat ear.

TABLE III. *The ability of various bacteria to induce ear thickening and acneiform lesions*

Bacteria[a]	Strain	Origin	Δ Thickness (mm × 100)[b]		Lesion count Day 15
			Day 7	Day 15	
P. acnes	UCLA 79	Inflammatory acne	90.3 ± 10.2[c]	96.5 ± 16.6	7.8 ± 1.8
P. acnes	CN 6134		74.4 ± 12.6	96.8 ± 13.9	7.6 ± 3.1
P. acnes	UCLA N1	Normal skin	79.4 ± 2.3	75.6 ± 7.6	5.4 ± 1.2
P. acnes	UCLA SC	Inflammatory acne	75.0 ± 5.0	95.8 ± 10.1	5.2 ± 1.1
P. acnes	UCLA N2	Normal skin	67.8 ± 5.5	61.2 ± 5.6	2.2 ± 1.1
P. acnes	UCLA LAN	Inflammatory acne	64.6 ± 6.3	57.2 ± 8.2	4.0 ± 1.7
P. acnes	UCLA N4	Normal skin	52.6 ± 5.0	64.8 ± 5.2	4.0 ± 1.0
P. acnes	UCLA N3	Normal skin	48.8 ± 7.1	46.6 ± 8.1	4.0 ± 2.1
P. acnes	UCLA 6S	Normal skin	17.8 ± 4.4	12.5 ± 4.7	0.2 ± 0.2
Staph. epidermidis	UCLA 1	Normal skin	63.0 ± 7.6	51.7 ± 6.6	0.2 ± 0.2
Strep. lactis	UCLA 2		11.0 ± 1.4	15.4 ± 5.6	1.0 ± 0.6
E. coli B	UCLA 3		20.0 ± 3.9	23.2 ± 4.2	0.0
Saline			3.0 ± 2.0	1.0 ± 1.8	0.0

[a] 140 μg bacteria injected on day 0.
[b] Δ Thickness = day n thickness − day 0 (preinjection) thickness.
[c] Mean ± SE.

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