RESEARCH ARTICLE

Importance of *Propionibacterium acnes* hemolytic activity in human intervertebral discs: A microbiological study

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Abstract

Most patients with chronic lower back pain (CLBP) exhibit degenerative disc disease. Disc specimens obtained during initial therapeutic discectomies are often infected/colonized with *Propionibacterium acnes*, a Gram-positive commensal of the human skin. Although pain associated with infection is typically ascribed to the body’s inflammatory response, the Gram-positive bacterium *Staphylococcus aureus* was recently observed to directly activate nociceptors by secreting pore-forming α-hemolysins that disrupt neuronal cell membranes. The hemolytic activity of *P. acnes* in cultured disc specimens obtained during routine therapeutic discectomies was assessed through incubation on sheep-blood agar. The β-hemolysis pattern displayed by *P. acnes* on sheep-blood agar was variable and phylogroup-dependent. Their molecular phylogroups were correlated with their hemolytic patterns. Our findings raise the possibility that pore-forming proteins contribute to the pathogenesis and/or symptomology of chronic *P. acnes* disc infections and CLBP, at least in a subset of cases.

Introduction

Chronic lower back pain (CLBP) is a leading cause of disability among adults worldwide. CLBP has been ranked the number one cause of years lived with disability in the United States [1], and substantial CLBP rates have been reported in Germany (27%) [2], Sweden (33.2%) [3], and Latin America (31.3%) [4]. Most patients with CLBP exhibit degenerative disc disease, an idiopathic condition that predisposes to herniation, sciatica, and significant morbidity/
disability. Regarding the underlying mechanism of pain, Freemont et al. [5] has demonstrated ingrowth of nerve fibers into degenerated discs (usually accompanied by the presence of annular fissures), representing a potential source of nociception in CLBP, although frank inflammation is often lacking within the paucicellular disc tissue.

In 2001, Stirling et al. [6] first published evidence suggesting a relationship between chronic bacterial infection and disc degeneration. A growing body of literature has since argued that disc specimens are often already infected/colonized with Propionibacterium acnes at the time of initial therapeutic discectomies (i.e. prior to any previous surgical manipulation). P. acnes is a prominent Gram-positive bacterial commensal of the human skin and pilosebaceous unit. In multiple independent studies from 2001 to 2017, P. acnes was isolated from 26% of resected specimens [7–11]. Two 2015 meta-analyses reported a pooled prevalence of bacteria at 34% [12] and 36.2% [13], with P. acnes as the predominant species. In a recent 368-patient study, Capoor et al. [14] confirmed these observations and directly visualized the presence of P. acnes biofilms in situ within resected discs. This result is most consistent with chronic infection.

Chronic infections are often associated with pain symptoms. This type of nociception is classically attributed to body’s inflammatory cascade, a cytokine-induced phenomenon that involves leukocyte infiltration and vascular dilatation. Nevertheless, more direct mechanisms of pathogen-induced pain have also been suggested. For instance, Chiu et al. [15] recently demonstrated that Staphylococcus aureus can directly activate nociceptor neurons through elaboration of a pore-forming toxin (α-hemolysin) that can generate action potentials in these cells through pathological ion influx. Beyond S. aureus, bacterial pathogens secrete diverse pore-forming proteins that facilitate cytotoxicity by targeting host-cell membranes [16]. They are often referred to as ‘hemolysins’ for historical reasons—in that erythrocyte lysis (i.e. β-hemolysis) is often observed when these organisms are cultured on blood agar—although these proteins can likewise form pores in other cellular targets (for instance, white blood cells and platelets [17]). In this context, it is noteworthy that hemolytic activity has been associated with cultured strains of P. acnes, including isolates from orthopedic joint infections [18].

Accordingly, we hypothesized that P. acnes also expresses pore-forming proteins during chronic intervertebral disc infection/colonization, directly interacting with sensory neurons and contributing to symptoms of CLBP. The current study explores this hypothesis by assessing the hemolytic activity of P. acnes strains obtained from three independent studies. In the first study, Capoor et al. [14] assessed the presence of P. acnes in degenerated disc tissue obtained from 368 patients who underwent microdiscectomies. Of these, 119 (32.3%) were positive for P. acnes by culture (>1 colony-forming unit (CFU)/g). Only the 38 P. acnes-positive disc tissue samples with >1000 CFU/g were examined in this study. In the second study, Rollason et al. [19] assessed the presence of P. acnes in degenerated disc tissue obtained from 64 patients diagnosed with lumbar disc herniation who underwent discectomy surgery. Disc samples obtained from 24 (38%) of these patients tested positive for P. acnes by culture (1–150 CFU per sample). We were able to obtain 67 separate P. acnes isolates recovered from these tissues for use in this study. In the third study, Albert et al. [20], assessed the presence of P. acnes in degenerated disc tissue obtained from 61 patients who underwent primary surgery at a single spinal level for disc herniation. Anaerobic cultures were positive in 26 (43%) of these patients. We were able to obtain P. acnes-positive disc tissue samples for this study.

**Materials and methods**

At the time of the three independent studies, Capoor et al. [14], Rollason et al. [19] and Albert et al. [20], each received Ethics Committee approval and the respective studies were approved.
by the Institutional Review Boards of the participating hospitals. Further, written informed consents were obtained from each patient participating in the respective studies.

β-Hemolytic activity of disc tissue and disc tissue homogenates

Homogenates (a total of 38) from disc tissue samples obtained during a previous study by Capoor et al. [14], along with several solid fragments of intervertebral disc tissue, were directly inoculated on the surface of Wilkins-Chalgren Anaerobe Agar containing 7% sheep's blood and vitamin K (Hi Media Laboratories) and then incubated at 37°C for 14 days under anaerobic conditions. Positive hemolysis was recorded if complete zones of clearance were observed on these plates.

Frozen intervertebral disc tissue samples excised from lumbar disc herniations by Albert et al. [20] were used to inoculate plates made using Columbia blood agar containing 7% (v/v) defibrinated sheep's blood. These plates were incubated as described above. Positive hemolysis was recorded if complete zones of clearance were observed.

β-Hemolytic activity and phylotyping of P. acnes strains isolated from disc tissue

Fifty-one colonies were selected visually (depending upon the hemolytic characteristics observed) from the 38 P. acnes-positive disc tissue homogenates studied by Capoor et al. [14]. Hemolytic activity was assessed using Columbia Blood Agar Base (Oxoid) supplemented with 7% sheep erythrocytes that had been washed with phosphate-buffered saline. These plates were incubated as described above. Positive hemolysis was recorded if complete zones of clearance were observed on agar plates. These samples were subjected to multiplex PCR typing to determine their phylogroups [21, 22]. Where a sample had both hemolytic and non-β-hemolytic colonies, more than one colony was selected for multiplex PCR typing.

A hemolytic P. acnes strain from the Collection of Microorganisms (Department of Microbiology, Faculty of Medicine, Masaryk University) isolated phylotype IB from clinical material was used as a positive control to evaluate hemolytic activity. For phylotyping of our isolates, we utilized Nagy et al.’s methodology [21], which we tested for accuracy against the set of clinical P. acnes isolates from the Collection of Microorganisms.

The sixty-seven P. acnes isolates obtained from Rollason et al. [19] had already been subjected to phylogroup analysis using recA sequencing and monoclonal antibody typing. Hemolytic activity was assessed using Columbia blood agar supplemented with 7% (v/v) defibrinated sheep’s blood. These plates were incubated as described above. Positive hemolysis was recorded if complete zones of clearance were observed on agar plates.

Identification of bacteria

Capoor et al. utilized matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) for identification of P. acnes, Rollason et al. utilized 16S rRNA-based PCR, and Albert et al. utilized (API) biochemical analysis using the Rapid ID 32A kit and by 16S rDNA PCR.

Results

β-hemolytic activity observed in cultured disc tissue homogenate

Homogenates from the 38 disc tissue samples available from the study by Capoor et al. [14] were used to inoculate blood agar plates that were subsequently incubated under anaerobic conditions. Fragments of disc tissue were also streaked over the surface of the blood agar plates before pressing and embedding into the agar surface for incubation. These samples could be
most representative of the spinal condition since they were fresh tissue samples excised from
patients and cultured the same day. Additional disc tissue samples from the study by Albert
et al. [20] were similarly used to inoculate blood agar plates. Photographs of representative
plates are presented in Fig 1.

After anaerobic culture, 26 (68%) of the 38 plates inoculated with homogenates contained
colonies that showed β-hemolysis (clearing of the blood agar) as well as 12 (32%) plates with
colonies that displayed no hemolytic activity. Some of the bacterial colonies (subsequently
identified as *P. acnes*) derived from disc tissue fragments showed β-hemolysis, while others
showed either no hemolysis or a brown color likely caused by porphyrins produced by *P.
acnes*. Of the 26 hemolytic samples, 21 had *P. acnes* as the only bacteria isolated from the disc,
and 5 samples had additional bacteria cultured (2 *S. haemolyticus*, 1 *S. pasteurii*, 1 *S. warneri*,
and one with both *P. granulosum* and *F. magna*). Of the 12 non-hemolytic samples, 10 had *P.
acnes* as the only bacteria isolated form the disc, and 2 had additional bacteria cultured (1 *P.
granulosum* and one *S. epidermidis*).

**β-hemolytic activity and phylogroup analysis of *P. acnes* strains isolated from disc tissue**

To assess the *in vitro* hemolytic properties of disc-derived *P. acnes* strains, archived isolates
from two previous clinical studies were assessed. The results are presented in Table 1.

![Image of hemolytic activity of *P. acnes* in infected intervertebral disc tissue.](https://doi.org/10.1371/journal.pone.0208144.g001)

### Table 1

| Phylogroup | No. of Samples |
|------------|----------------|
| *P. acnes* | 41             |
| *S. haemolyticus* | 2               |
| *S. pasteurii* | 1               |
| *S. warneri* | 1              |
| *P. granulosum* | 1               |
| *F. magna* | 1              |
| *S. epidermidis* | 1               |
A total of 51 morphologically distinct colonies were cultured from the 38 disc specimens obtained from the study of Capoor et al. [14] Of note, whenever a single disc specimen yielded both β-hemolytic and non-hemolytic colonies, all colony types from that specimen were selected for inclusion here. Multiplex PCR typing was performed to assign the colonies to the appropriate phylogroup. For a majority of these samples, only the visibly dominant strain’s phylogroup was reported. As summarized in Table 1 and broken down according to phylogroup, 26 (51%) of the 51 strains were β-hemolytic (51%). The vast majority of the isolates from phylogroup I (including subgroups IA, IB and IC) were β-hemolytic, but all of the isolates from phylogroup II were non-hemolytic. No isolates from phylogroup III were recovered. The hemolysis pattern from the disc homogenates (Fig 1) corresponded 100% to the hemolysis pattern of the P. acnes strains subsequently cultured from the same homogenates.

From the independent study of Rollason et al. [19], 67 additional disc-derived isolates of P. acnes, which had been assigned phylotypes in the original study, were obtained for hemolysis profiling. These isolates were included here irrespective of their original quantitative abundance from disc cultures. As summarized in Table 1, 20 (30%) of the 67 isolates were β-hemolytic. As seen with the samples from Capoor et al.’s study [14], the vast majority of the samples from phylogroup I (IA and IB, in this dataset) were β-hemolytic, while all of the strains from phylogroup II were non-hemolytic. Seven strains from phylogroup III were present in this sample set, and all of them were also found to be non-hemolytic.

### Discussion

A substantial portion of the P. acnes strains tested in this study (46 [39%] of 118) displayed β-hemolytic activity in culture. This is higher than the percentage observed by Wright et al. [24], who noted that 22 (21%) of 106 P. acnes strains obtained from sterile body sites exhibited hemolytic activity with sheep blood. However, it is a lower percentage than that observed by Nodzo et al. [18], who found that 13 (59%) of 22 P. acnes strains isolated from patients who had undergone shoulder arthroplasty exhibited hemolytic activity. Follow-up studies by Boyle et al. [25] and Mahylis et al. [26] found that 16 (52%) of 31 and 20 (51%) of 39, respectively, isolates from patients who had undergone shoulder arthroplasty exhibited hemolytic activity. When the results of all these studies are added together, 37% of clinical P. acnes isolates have exhibited hemolytic activity, suggesting that the results of this study are typical of P. acnes strains isolated from samples obtained in surgical settings.

In healthy adult humans and animals, nerves do not extend beyond the outer third of the annulus fibrosus. However, Freemont et al. [5] have demonstrated the presence of nerves, including those that express the Substance P (a nociceptive neurotransmitter), in the inner third of the annulus fibrosus, the nucleus pulposus, or both, of patients with chronic low back pain. Infection of these tissues with P. acnes would place the infecting bacteria in proximity of

| Phylogroup | Number of isolates | Number (%) positive for β-hemolysis | Number of isolates | Number (%) positive for β-hemolysis |
|------------|--------------------|-------------------------------------|--------------------|-------------------------------------|
| IA1        | 20                 | 16 (80%)                            | 19                 | 16 (84%)                            |
| IB         | 10                 | 9 (90%)                             | 6                  | 4 (67%)                             |
| IC         | 1                  | 1 (100%)                            | 0                  | -                                   |
| II         | 20                 | 0 (0%)                              | 35                 | 0 (0%)                              |
| III        | 0                  | -                                   | 7                  | 0 (0%)                              |
| Total      | 51                 | 26 (51%)                            | 67                 | 20 (30%)                            |

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these ingrown nociceptive nerves, raising the potential for an interaction between \textit{P. acnes} and nociceptive neurons that results in pain (Fig 2). Chiu \textit{et al.} \cite{15} have relatively recently shown, using a mouse model, that \textit{S. aureus} induces calcium flux and action potentials in sensory neurons that modulate inflammation through the secretion of N-formylated peptides and the pore-forming toxin \textit{α}-hemolysin. Mechanical and thermal hyperalgesia in these mice correlated with live bacterial load rather than with tissue swelling or immune activation. Thus, a reasonable mechanism by which chronic \textit{P. acnes} infection of one or more intervertebral discs might induce CLBP is the ability of \textit{P. acnes} to secrete pore-forming toxins like the \textit{α}-hemolysin of \textit{S. aureus} (Fig 2B). The microbiological findings presented here confirmed that (pore-forming) hemolytic factors are associated with disc tissue, disc homogenates, and \textit{P. acnes} isolates obtained from patients undergoing discectomy. These findings support the hypothesis that chronic low back pain may, in some cases, be associated with \textit{P. acnes} infection of the intervertebral disc and that this pain may correlated with live bacterial load.

The concept of direct microbe-mediated nociceptor stimulation rather than biomechanical problems being the mode of CLBP-generation would bridge several logical gaps. For one, it would explain how an “undercover” infectious colonization of the very particular physiological and biochemical environment within degenerative human discs could lead to low back pain presenting a clinically obvious pyogenic infection, as would be seen with other pathogens. In addition, the discovery of a dedicated nervous supply to the vertebral body and to the endplates not only provides explanations for how vertebroplasty can reduce fracture-related back pain—it also offers an explanation for how inflammatory endplate-related changes of adjacent bone marrow (Modic Type 1 changes) might correlate with low back pain. A dense nociceptive innervation of the vertebral endplate and the histochemical finding of Substance P in the related afferences \cite{27–29} clearly indicate that not only secondary ingrowth of nerve fibers into degenerative annulus defects but also the vertebral endplate itself are potential targets for such a pain-generating mechanism. An early interventional study \cite{30} demonstrated that so-called discogenic pain can be treated by interruption of the basivertebral nerve. All these data support the need for more research and a better understanding of what chronic low back pain really is and which pathophysiological processes are the most frequent causes.

Phylotyping of the \textit{P. acnes} isolates obtained from resected disc tissues showed that isolates from phylogroups II and IA were dominant, while isolates from phylogroups IB, IC and III were present in much smaller numbers. Although these results are somewhat different from the initial observations of McDowell \textit{et al.} \cite{22}, who observed a much larger proportion of phylotype III isolates, the results presented here were obtained using a much larger number of disc tissue samples. Hemolytic activity was restricted to those isolates from phylogroup I, including phylogroups IA, IB, and IC; the substantial number of phylogroup II isolates and the small number of phylogroup III isolates showed no hemolytic activity (Table 1). These results are consistent with those of McDowell \textit{et al.} \cite{31} and underline the need for more detailed study of the potential pore-forming toxins of \textit{P. acnes} and their phylogroup distributions.

On a true etiologic level, the presence of a \textit{P. acnes} toxin (even within a disc) is not complete evidence that it is performing the physiologic function we hypothesize. \textit{In vitro} experiments, in which \textit{P. acnes} culture supernatant (or recombinant pore-forming exotoxin) could be applied to a neuronal tissue culture model, are needed to examine the effect of \textit{P. acnes} proteins on depolarization. Identifying the genes responsible for the ability of \textit{P. acnes} to form pores in neuronal membranes would require knock-in/knock-out experiments with putative hemolysin genes. Mutagenesis models of \textit{P. acnes} CAMP genes have been developed \cite{32}, and a similar model could be applied to generate \textit{P. acnes} hemolysin knockouts, which could be evaluated using designs analogous to those used in the previous animal model studies \cite{15}. Ultimately, such strains might also be investigated in an animal model of \textit{P. acnes} discitis \cite{33].
Importance of *Propionibacterium acnes* hemolytic activity in human intervertebral discs

A

**DDD (Generally Accepted Disc Degeneration Mechanism)**

- Unknown Genetic or Environmental Catalyst
- IL-1β & other Cytokines
- Proteolytic Enzymes
- ECM Damaged (fragmented collagen, aggrecan, and hyaluronic acid)
- Neurotrophins (NGF and BDNF)

↓ nerve ingrowth

Nucleus Pulposus

Annulus Fibrosis

B

**DDD with CLBP (Hypothesized Pain Mechanism)**

- *P. acnes*
- Other pore-forming factors
- Pore-forming/hemolytic factors

↓ nerve ingrowth

Mechanical nociceptor

Thermal nociceptor

Nucleus Pulposus

Annulus Fibrosis
Such a model could be established to assesses “pain” and not simply proliferation of bacteria within the disc.

To investigate when and how hemolysin genes are secreted, the elaboration of pore-forming exotoxins should be studied under diverse growth conditions (e.g. planktonic exponential, planktonic stationary, surface-associated biofilm). Protein expression within a disc may be different from all growth conditions tested, and resected disc tissue would need to be evaluated to represent in situ conditions. In addition, clinical studies could be proposed in patients undergoing microdiscectomy for disc herniation. Validated microbiological detection of *P. acnes* in the extracted disc tissue would be pursued with the two principal aims being: (i) evaluation of the association between *P. acnes* hemolytic activity in the infected disc tissue and pre-operative pain scores, and (ii) determination of the pore-forming factors expressed in the disc tissue specimens and evaluation of their association with pre-operative pain scores.

The presence of genetic code for putative hemolysins in virtually all known *P. acnes* genomes, as well as the hitherto not well-appreciated formation of hemolytic zones on microbiological culture plates growing *P. acnes* from human discs, should make spinal researchers think deeper and with renewed curiosity. It will be of great importance to test disc material that is obtained during surgery for hemolytic activity in a larger, prospective series. With regard to the suggested use of fresh tissue, a possible limitation of the current study is that it utilized frozen intervertebral disc tissue. If the initial observations presented here were to hold true in these series, such observations would support the pathogenicity of (low-grade infected) nucleus material. It would help explain why in many patients, nucleus fragments that do not exert a significant mechanical compression on the affected nerve root on imaging can still cause such intense radiculopathy.

Future studies could employ hemolytic assays directly on disc tissue material, proteomics using mass spectrometry on the crude disc tissue homogenate to assess whether hemolysins are expressed, mass spectrometry on liquid culture supernatants for secretome analysis, and qRT-PCR or RNA-seq for expression analysis. Hemolysin gene secretion, including assessment of the type I secretion system that does not require signal peptide, could also be studied.

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