

PROPIONIBACTERIUM ACNES AS AN IMMUNOSTIMULANT

A Senior Thesis

By

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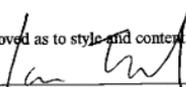
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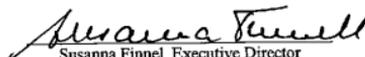
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ABSTRACT

The use of *Propionibacterium acnes* as an immunostimulant has been documented in various in vivo studies. Used frequently in the equine industry as a prophylactic treatment against Equine Respiratory Disease Complex (ERDC), *P. acnes* plays a role in activating the immune system through heightened macrophage function. While this activation is acknowledged, little is known about the specific pathway through which *P. acnes* act to bring about this activation. In an attempt to understand the mechanism of macrophage activation by *P. acnes*, studies were carried out using the macrophage cell line RAW 264.7. Treatment of RAW 264.7 cells with *P. acnes* in the presence of lipopolysaccharide (LPS) caused the activation of macrophages as observed by morphological changes. It also caused the induction of the inducible nitric oxide synthase (iNOS) in these cells. This induction of iNOS was dose-dependent and synergistic with LPS.

INTRODUCTION

Immunostimulation is a rapidly growing area in veterinary medicine. The United States Department of Agriculture has recently licensed numerous products for veterinary use that are directed toward nonspecific stimulation of the immune system. EqStim is one such product, manufactured by ImmunoVet, Inc. of Tampa, Florida (1).

EqStim is a 0.4 mg/mL nonviable *Propionibacterium acnes* suspended in 12.5% ethanol in saline. Given intravenously, EqStim stimulates a rapid activation of the macrophages resulting in the production of a variety of cytokines (1). Macrophages are known to play a key role in the immune response. Use of *P. acnes* as an immunostimulant has been shown to influence the major components of the immune system: macrophage activation, subsequent cytokine production, and enhanced activity and proliferation of B cell and T cell lymphocytes (1). The nonspecific immune stimulation by *P. acnes* results in activation of macrophages and the production of cytokines by the macrophages and immune cascade that they initiate (1).

Propionibacterium acnes.

Propionibacterium acnes are common bacterial flora of the skin, typically found within the deeper recesses (2). Acne inflammation begins with non-inflamed microcomedones, which eventually rupture, releasing the microflora of the follicle. *P. acnes* is thought to be the main component of this microflora. Extensive research has been done on *P. acnes*, not only in relation to acne models, but also for its unusual ability

to stimulate the reticuloendothelial system (RES) (3). The resistance of *P. acnes* cell wall to digestion, and subsequent persistence of phagocytized bacteria within the macrophages appears to be key in this ability (3,4). This stimulation has been demonstrated by enhanced clearance of particles from the bloodstream, resistance to viral infection, and resistance to tumor growth (5,6,7). Furthermore, it has been shown that intact, whole killed organisms are required for macrophage activation (8), in contrast to isolated components of other immunomodulator such as LPS (9). The component of *P. acnes* that is responsible for the activation of macrophages must be present in the immune system in the structural arrangement that is found in the intact organism. Further, it has been described that removal of the active component or disruption of the organisms' structure results in the loss of its ability to activate macrophages (8). Recognition and phagocytosis of *P. acnes* by macrophages triggers a series of biochemical events which leads to macrophage activation. An inability to effectively degrade this whole bacterium is potentially the cause of a persistent activation signal, hence prolonged macrophage activation (7).

Macrophages.

Macrophages have been found to play a key role in the early stages of wound healing (10). The importance of macrophage activation has been noted by many (11-15), resulting in a great number of studies undertaken in an attempt to stimulate macrophage activation, hence wound healing. Macrophage activation, as shown by Hunt *et al* (16), was seen to induce increased angiogenesis, fibroplasia, and collagen synthesis, important stages in the process of wound healing (15). Numerous immunomodulators have been

studied for their abilities to activate macrophages. Such activation, leading to increased wound healing, would be of significant benefit to the medical community.

Capable of repeated phagocytosis of foreign material, macrophages are responsible for the processing and presentation of foreign material for an immune response. Other functions include the release of cytokines, along with various other mediators that amplify an immune response, inflammation control, and the removal and repair of damaged tissues (12).

Macrophages are round cells, with a single nucleus. They are strongly adherent to glass/plastic surfaces. This anchorage dependence, attachment to and some degree of spreading on, is required for cell culture proliferation (17). This spreading is accomplished by long cytoplasmic filaments which are extended out from the body of the cell.

Normally found as “resting” macrophages, activation occurs upon exposure to inflammatory stimuli in inflamed tissues. Activated macrophages show heightened levels of phagocytic and secretory abilities, and are now known as “inflammatory” macrophages. Further, stimulation by bacterial products, such as *P. acnes*, results in “activated” macrophages (12).

Macrophages that have been activated in such a fashion, usually become enlarged. They tend to show increased membrane activity, especially seen as increased pseudopod formation. Activated macrophages are also seen to move much more rapidly than their unstimulated counterparts, in response to chemotactic stimuli. Activation also seems to impart an enhanced ability to kill intracellular organisms and tumor cells (18). Together,

the effects of activation allow macrophages a significantly increased effectiveness in the defense of the immune system (12).

A key reason attributed to their increased effectiveness lies in the generation of reactive nitrogen metabolites. Activated macrophages produce large quantities of nitric oxide. Nitric oxide is regarded as the effector molecule responsible for the tumoricidal and microbicidal activity of activated macrophages. Typically, a synergistic combination is required for maximal induction of the enzyme NO synthase (iNOS), which converts arginine and oxygen into citrulline and NO. However, once iNOS synthesis has occurred, prolonged, high production of NO is possible (5,9,19).

The many changes seen in activated macrophages allow for their increased microbicidal activity. Activated macrophages are thereby capable of a non-specific, cell-mediated immune response. Such non-specificity enables activated macrophages to phagocytose and destroy normally resistant organisms (12).

The purpose of this research was to study *in vitro* the activation of macrophages by *P. acnes* using a macrophage cell line RAW 264.7 .

MATERIALS AND METHODS

Reagents and cell culture.

The mouse macrophage cell line RAW 264.7 cells (Abelson leukemia virus transformed monocytic macrophage cell line) was obtained from the American Type Culture Collection (Rockville, Maryland). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island), which was supplemented with high glucose, L-glutamine, 110 mg/L sodium pyruvate, 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island), and 1% (v/v) penicillin (10000 U/ml) / streptomycin (10000 U/ml) (P/S) (Gibco BRL). All cell culture reagents were obtained from Life Technologies (Grand Island, NY). *Propionibacterium Acnes*, Immunostimulant (Serial # 670), consisting of 0.4 mg/mL nonviable *Propionibacterium acnes* suspended in 12.5% ethanol in saline, was obtained from ImmunoVet, Inc. (Tampa, Florida). Bacterial LPS (*Escherichia coli* serotype 0111:B4) was obtained from Sigma Chemical (St. Louis, MO).

Macrophage activation.

RAW 264.7 cells (1×10^6) were seeded into 60-mm tissue culture dishes and incubated overnight at 37 °C in 5% CO₂ in air to allow for adherence. Cells were incubated with varying dilutions of *Propionibacterium acnes*, Immunostimulant for 4 hours. At the end of the incubation period, the stimulus was removed, and replaced either with fresh medium or 10 ng/ml LPS. After a further incubation period of 48 hours, the medium was removed for use in the nitric oxide measurement. Cells were then rinsed twice with 1X PBS before being scraped off the plate in 250 µl of lysis buffer (10 mM Tris-Cl pH 7.5), 1% SDS) that had been heated to 80 °C prior to adding. Cell lysates

were transferred to sterile eppendorf tubes using a 21.5 gauge needle. They were further incubated at 80 °C for five minutes and then cooled on ice before being stored at 4 °C.

Assay for NO synthesis.

Synthesis of NO was determined by assay of culture supernatants for NO²⁻, a stable reaction product of NO with molecular oxygen. Briefly, 50 µl of culture supernatant was incubated with an equal amount of Greiss reagent [0.5% sulfanilamide, 0.05% N-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% H₃PO₄] in a 96-well tissue culture plate for ten minutes at room temperature. The amount of colored product was determined spectrophotometrically at 570 nm in an automated ELISA reader (MR 600, Dynatech, Torrance, CA). The amount of nitrite was estimated, according to the standard curve generated using known concentrations of sodium nitrite. Results are presented as nmol NO/µg protein/48 hours.

Protein estimation.

The BCA Protein Assay (Pierce, Rockford, IL) was used for the spectrophotometric determination of protein concentration.

Cell staining.

To determine the effects of *P. acnes* and LPS on macrophage morphology, RAW 264.7 cells were allowed to adhere in each chamber of two-chamber Lab-tek culture slides (Nunc, Naperville, CT). Adherent macrophages were then cultured for 4 hours

with varied dilutions of *P. acnes*. Following this incubation, medium was removed and replaced either with fresh medium alone or 10 ng/ml LPS.

Western blot.

Cell monolayers were washed with ice-cold PBS and lysed in buffer containing 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium phenylmethylsulfonyl fluoride. SDS-PAGE (10%) was conducted under denaturing, reducing conditions according to Laemmli (22). Proteins were transferred onto 0.2- μ m-pore-sized polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) using 15% methanol, 25 mM Tris, and 192 mM glycine, pH 8.3. The membrane was blocked for 1 hour at room temperature with 1% bovine serum albumin in Tris-buffered saline (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) and then incubated with mouse anti-mac-NOS monoclonal antibody (1/500 dilution; Transduction Labs) for 1 hour at room temperature. The membrane was washed with Tris-buffered saline and then subsequently incubated for 1 hour with anti-mouse IgG conjugated to alkaline phosphatase (Sigma Chemical). After washing, the membrane was equilibrated in alkaline phosphatase buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl₂) and developed in a solution of 167.5 μ g/ml nitro blue tetrazolium and 167.5 μ g/ml 5-bromo-4-chloro-3-indoyl phosphate in alkaline phosphatase buffer.

RESULTS

Effects of *P. acnes* and LPS on macrophage morphology.

When cultured in medium alone, normal RAW cells tend to be round in shape and have small, lobular nuclei. No spreading is evident (Figure 1A). RAW cells exposed to *P. acnes* alone appeared to have moderate cytoplasmic spreading and slight vacuolation (Figure 1B). RAW cells exposed to LPS appear to have slight spreading and vacuolation (Figure 1C). In contrast, RAW cells exposed to both LPS and *P. acnes* were greatly enlarged. They appear highly vacuolated with increased cytoplasmic spreading (Figure 1D). Per field, activation (Figure 1D) is proportionately elevated in comparison to the figures previously described (Figures 1A, 1B, 1C).

The effects *P. acnes* and LPS on nitric oxide production.

As shown in Figure 2, RAW cells release a limited amount of nitric oxide in response to medium alone. When exposed to *P. acnes*, the cells exhibit a dose dependent increase in nitric oxide production. RAW cells exposed to LPS alone show a significantly increased production of nitric oxide. However, an even more significant increase is shown in RAW cells exposed to both LPS and *P. acnes*, again exhibiting a *P. acnes* dose dependency. *P. acnes* (1:1000) and (1:10,000) dilutions showed the greatest increase in nitric oxide production over LPS alone. This suggests that more highly activated macrophages were obtained from RAW cells treated with LPS and *P. acnes* in combination.

The effects of *P. acnes* and LPS on total protein content.

As shown in Figure 3, total protein content of RAW cells in medium alone, as well as those treated with varying doses of *P. acnes*, exhibit similar concentrations. A significant increase in protein concentration is seen in control cells treated with LPS alone. However, in those RAW cells treated both with *P. acnes* and LPS, an even larger increase in protein concentration can be seen in 1:10 and 1:100 dosages. The *P. acnes*/LPS combination protein concentrations are then reduced in 1:1000 and 1:10,000 *P. acnes* dosages.

Western blot analysis of iNOS.

P. acnes in the presence of LPS induced the expression the iNOS protein. Treatment of RAW cells with *P. acnes* in the presence of LPS for a period of 48 hours induced the expression of the iNOS protein (Figure 5). This expression was not dose dependent. However, treatment of cells with LPS alone or *P. acnes* (1:10) alone, also induced expression to an equal extent. Low levels of iNOS protein were observed in cells treated with media alone, as well as in those treated with higher dilutions of *P. acnes*.

The effects of LPS and Interferon- γ on RAW cells.

In preparation for the previous experimentation, the RAW cell activation model using LPS and IFN- γ developed by Nathan, et. al. (9,19), was reproduced with similar results, as seen in Figure 4. The reproduction of these results allowed for the basic understanding of techniques involved. Furthermore, this model provided an excellent

basis upon which to build the *P. acnes* model, due to its similar nature and proficiency in describing the pathway of LPS activation. These results proved useful for comparison to those of *P. acnes* experimentation.

DISCUSSION

Used extensively in equine medicine, EqStim has been shown to be effective in the treatment of Equine Respiratory Disease Complex (ERDC) (20). Administered prophylactically, a significant decrease in the incidence of stress-induced ERDC was shown in horses during and following transportation. Prophylactic use of immunostimulants has been shown to be effective in enhancing nonspecific resistance to viral infection and tumor metastasis (7). Research by A. Nevsted has indicated that horses are at increased risk of respiratory infections during shipping, due to stress placed on the animal. This stress leads to the “down-regulation” of the animal’s immune system and makes them more susceptible to infections. By activating the immune response (i.e. macrophage activity) prior to pathogen exposure, there is a potential to enhance a horse’s ability to withstand such a challenge (20). Further studies by Nevsted have indicated that protection against illness is based upon the short term (9-14 days) stimulation of cell mediated immune response.

Economically, respiratory disease in horses can prove extremely costly to owners in both down time and in antibiotic treatment. The use of products such as EqStim can prove extremely useful and cost effective on a prophylactic basis. The cost of this immunostimulant is minimal in comparison to the costs associated with treatment of a sick horse. In addition, *P. acnes* used in addition to conventional antibiotic therapy has been shown to accelerate the recovery rates in acute and chronic ERDC cases by as much as 60%. The multiple-dose regime of *P. acnes* also allows for a greater flexibility in treatment of the patient, according to individual clinical improvement (21).

The symptoms of ERDC seen commonly in equine practices include nasal discharge and cough syndrome. Antibiotics are normally effective against bacterial pathogens, however, if the horse is immunodeficient, these symptoms will recur even following a prolonged antibiotic treatment regime. In addition, antibiotics prove ineffective against a viral component involved in ERDC infections. Many veterinarians have come to rely on EqStim for the treatment of horses exhibiting symptoms such as these. Administration of a dose of EqStim during the initial visit, together with antibiotic therapy has been shown to shorten the recovery time as compared to using antibiotics alone. In summary, "Immune stimulation, through stimulation of macrophage activities, provides a rational basis for treatment of any animal disease where the immune system gives evidence of sub-optimal function" (21).

P. acnes products have a broad range of usage, not limited merely to ERDC. Off label studies include the treatment of osteomyelitis, chronic endometritis, and wounds in horses. The treatment of tumors, feline leukemia cats, canine parvo infection, demodectic mange, and canine/feline respiratory disease has also benefited from the use of *P. acnes* immunostimulation (1). With such profound results shown in *in vivo* studies, the experimentation previously described was undertaken in order to better understand the pathways of macrophage activation by the EqStim immunostimulant, *P. acnes*.

While there was a significant amount of literature pertaining to the activation of macrophages, the vast majority was carried out using *in vivo* studies. Additionally, very little correlation could be found among the publications. It appeared that each had followed a separate idea, none building upon the findings of another as is evident in LPS studies of a similar nature. Therefore, it was difficult to follow the progress made in

determining the signal transduction pathways involved in the activation of macrophages by *P. acnes*.

The signal transduction pathway involved in the activation of macrophages by LPS is extremely well described, having been determined in a methodical and progressive nature (9). This model of macrophage activation by LPS was used as a basis for performing the experiments with *P. acnes*.

P. acnes in the presence of LPS caused the activation of macrophages as observed by the morphological changes. Immunologically activated macrophages produce NO, the reactive free radical that mediates the bactericidal and tumoricidal activities of macrophages. *P. acnes* in the presence of LPS also caused an increase in the production of nitric oxide in these cells. This increase in NO production was dose dependent and synergistic with LPS. However unlike the LPS stimulation where there was an increase in NO production with increasing concentrations of LPS, higher concentrations of *P. acnes* caused a decrease in NO production. Moreover there was no dose dependent increase in the expression of the iNOS protein. These results indicate that although both LPS and *P. acnes* cause the activation of macrophages, they might be doing this by two different pathways. Induction of iNOS by LPS involves NF κ B, an interferon response element (24). It would be interesting to see if these transcription factors are also involved in the action of *P. acnes*.

EqStim is used rather sparingly in horses, recommended dosages being 1 ml per 250 lbs. of body weight in mature horses (21). Producing only a mild rise in temperature, tremors, and inappetence up to 24 hours post administration, this product is generally regarded as safe (1). The concentrations used in these experiments were proportionately

greater than would normally be found in the body system. However it is difficult to compare results from this study on a transformed cell line with those from *in vivo* studies. The next logical step to be taken would be to apply this *P. acnes* model to mouse macrophages *in vitro*, and then possibly to take those findings to an *in vivo* mouse study.

By progressively building upon these results, it is hoped that the *P. acnes* activation pathway may one day be developed as fully as that of LPS. Such knowledge could prove extremely beneficial not only in furthering the treatment of animal infections, but in the realm of human medicine, as well. The capacity to therapeutically manipulate macrophage function holds great potential for the medical community, at large.

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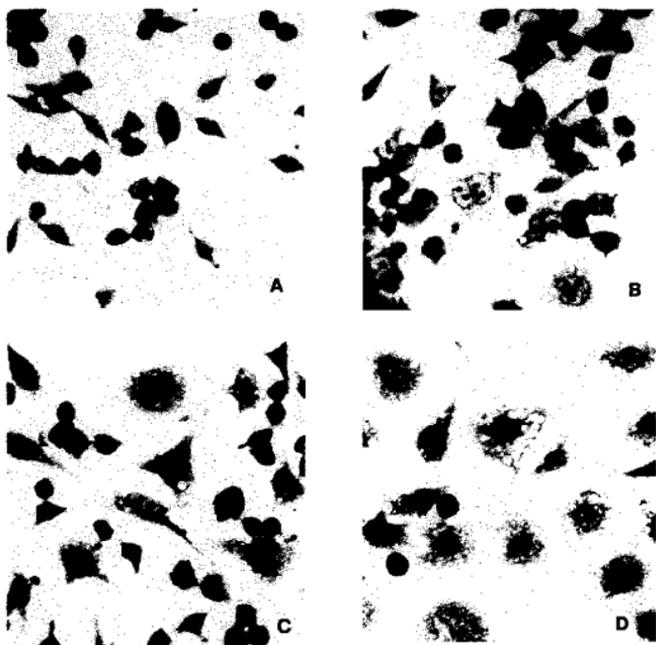


Figure 1. Macrophage morphological changes in response to *Propionibacterium acnes*. RAW cells were cultured on two-chamber Lab-tek culture slides in medium alone (A), 10 ng / ml LPS (B), 1:10 *P. acnes* (C), and the combination (D) for 24 hours.

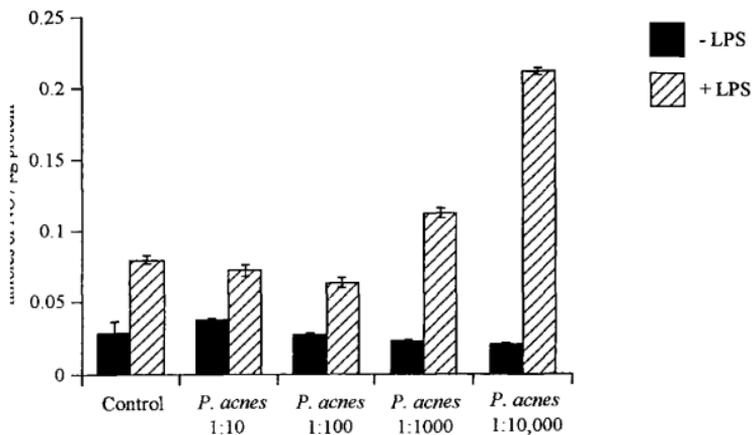


FIGURE 2. The effects of Propionibacterium acnes and Lipopolysaccharide on Nitric Oxide production.

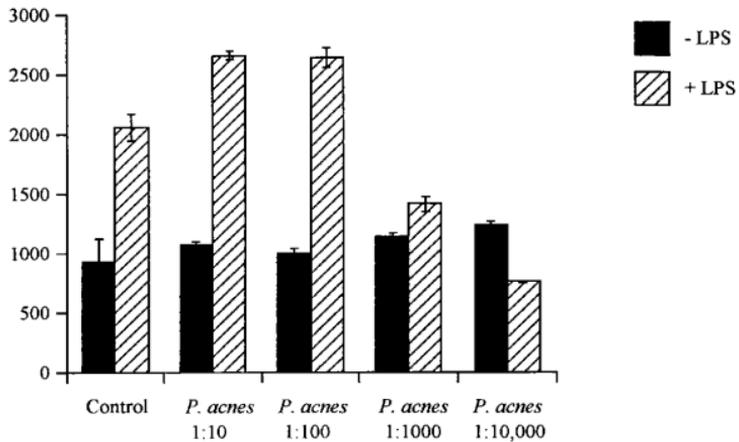


FIGURE 3. The effects of Propionibacterium acnes and Lipopolysaccharide on total protein concentration.

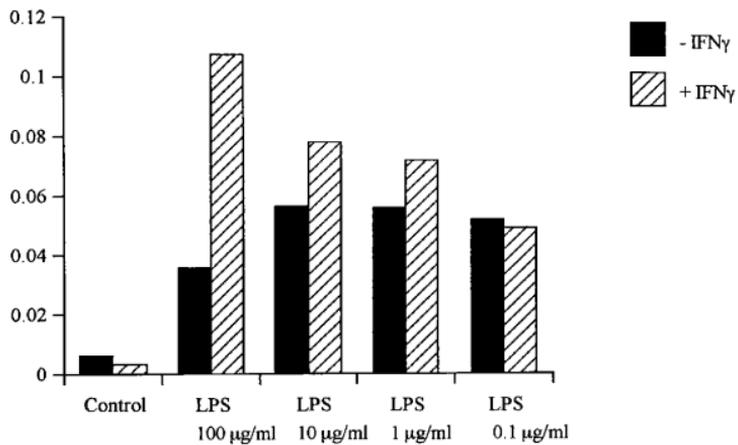


FIGURE 4. The effects of Lipopolysaccharide and Interferon-gamma on RAW cells.



FIGURE 5. Dose dependent analysis of induction of iNOS protein by *Propionibacterium acnes* and Lipopolysaccharide (LPS). RAW 264.7 cells were incubated in varying dilutions of *P. acnes* in the presence or absence of LPS (10 ng/ml) for 24 hours. Total cell lysates were subjected to SDS-PAGE (10%), blotted onto a polyvinylidene difluoride membrane, and probed with anti-mac-NOS antibody. Lane 1 represents the migration of the molecular weight markers, lane 2 is the positive control for iNOS (Transduction Labs, Inc.), lanes 3-6 represents LPS (10 ng/ml) + *P. acnes* (1:10,000, 1:1,000, 1:100, 1:10), lane 7 represents LPS alone, lanes 8-11 represents *P. acnes* (1:10,000, 1:1,000, 1:100, 1:10), and lane 12 represents treatment with medium alone. The iNOS band is at 130 kDa.