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Type-I Hypersensitivity to *Malassezia pachydermatis* Extracts in Healthy Dogs and Dogs with *Malassezia* Otitis Externa

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Abstract : The purpose of the study reported here was to test the hypotheses that clinically healthy dogs will not manifest immediate hypersensitivity responses to intradermal injection of *Malassezia pachydermatis* extracts but that affected dogs with *Malassezia* otitis will manifest such hypersensitivity. We desired to identify approximate molecular mass of any allergenic components of the yeast by use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein profile of *Malassezia pachydermatis* extracts showed between 16 and 110 kDa. Especially, the intensity was strongest between 25 and 80 kDa. Mean wheal diameters in the affected groups of 20, 2, 0.2, and 0.02 μg/ml were 13.36 ± 0.67, 5.33 ± 0.67, 5.47 ± 0.82, and 5.07 ± 0.64, respectively. Mean wheal thickness in the affected groups of 20, 2, 0.2, and 0.02 μg/ml was 6.44 ± 0.40, 3.86 ± 0.35, 2.64 ± 0.36, and 2.60 ± 0.44, respectively. The difference of wheal diameters and thickness between healthy and affected groups was significant ($p < 0.05$). In conclusion, the observations confirm that *Malassezia pachydermatis*-derived antigens may induce an immediate wheal response when intradermal injected in dogs. It seems reasonable to suggest that hypersensitivity to yeast may contribute to the development of clinical signs in dogs with immediate skin test reactivity, especially in dogs with *Malassezia* otitis externa.

Key words **:** *Malassezia pachydermatis*, hypersensitivity, otitis externa, dogs.

Introduction

The lipophilic yeast *Malassezia pachydermatis* is part of the normal cutaneous microflora of most warm-blooded vertebrates. It is now widely accepted that *Malassezia pachydermatis* is an opportunistic pathogen of the dog, with an important role in both otitis externa (12) and dermatitis (10). The presence of high population densities of the yeast in lesional skin in dogs with dermatitis refractory to antibacterial and anti-inflammatory therapy, and the clinical response following antifungal treatments and the associated reduction in yeast numbers provide good evidence for a pathogenic role (2,10,21).

Much less has known about immunity to skin colonization and infection by yeasts of the genus *Malassezia*. Abnormal cell-mediated immune responses have been reported in people with seborrheic dermatitis associated with lipid-dependent *Malassezia* species (30). Hypersensitivity responses to *Malassezia* antigens have been reported in people with the head-neck form of atopic dermatitis (15).

In recent years, skin disease associated with *Malassezia pachydermatis* has been commonly recognized in dogs (10,18, 21), and the population density of the yeast has been shown to be markedly higher in areas of the skin with lesions (2,3).

Several factors have been suggested to cause overgrowth of the organism, such as alterations in skin surface microclimate and defective host immunity (22). These factors may be associated with various underlying skin diseases including hypersensitivity disorders, bacterial skin infections, keratinization defects and endocrine diseases (21,22,25,27).

Atopic dermatitis is one of the most common diseases associated with *Malassezia* overgrowth in dogs. It has been demonstrated that some atopic dogs carry higher numbers of *Malassezia pachydermatis* on the skin than normal dogs (1,6,32) and cytological evidence of *Malassezia* overgrowth is a common finding in atopic dogs.

Wide variability in the number of *Malassezia* organisms that can be isolated from the skin of healthy dogs has been noted. Variation according to anatomic site within individual dogs is significant (5,16). These factors have been a hindrance to establishing reference range values for the number of yeast organisms to be expected on healthy dogs. In addition, the anatomy of the canine ear predisposes the dog to microbial proliferation that may result in clinical diseases. The external canal is long and narrow and turns medially at the junction of the vertical and horizontal segments. As moisture content increases, the stratum corneum becomes progressively macerated decreasing the protective mechanism and providing a medium for organism proliferation (34).

Otitis externa of the dogs is a major inflammatory disease of the external ear canal inflicting as much as 20% of the canine population (26,34). Although it is a common disorder,

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etiological and predisposing factors and the role of microorganisms in the pathogenesis still remain uncertain. Etiological factors of otitis externa have not been clearly identified and numerous factors are implicated either as primary initiating causes or secondary complications (26,33,34).

In more recent studies, *Malassezia pachydermatis* was commonly isolated from clinical cases of otitis externa (7,9,11,19, 29,31). *Malassezia pachydermatis* was isolated in 80% of otitic ears and 21% of normal ear (19), and 72% and 36% (7), and 57% and 17% (11), respectively. Mansfield *et al* (20) investigated the role of *Malassezia pachydermatis* as a pathogen and the effects of moisture and yeast byproducts in the normal external ear canal of dogs and reported that *Malassezia pachydermatis* is an opportunistic pathogen. In our knowledge, despite of a strong association between *Malassezia pachydermatis*-induced hypersensitivity and otitis externa, there have been no reports.

The purpose of the study reported here was to test the hypotheses that clinically healthy dogs will not manifest immediate hypersensitivity responses to intradermal injection of *Malassezia pachydermatis* extracts but that affected dogs with *Malassezia* otitis will manifest such hypersensitivity.

Materials and Methods

Animals

Five healthy dogs and five dogs with *Malassezia* otitis were tested intradermally with extracts prepared from *Malassezia pachydermatis* isolates. The diagnosis of *Malassezia* otitis was based on the presence of relapsing greasy, erythematous otitis affecting the external canal, accompanied by large populations of the yeast in representative lesions as assessed by cytologic examination. Dogs were determined to be clinically normal on the basis of results of complete physical and dermatologic examinations. *Malassezia* overgrowth was diagnosed by microscopic observation of modified Wright stain (Diff-Quik, Dade-AG, Switzerland) stained tape strips. Samples were obtained from the groin, axilla and interdigital web and *Malassezia* overgrowth was characterized as an average of 5 or more *Malassezia* organisms per 400 × field (23). Involvement of *Malassezia* in cases of otitis externa was assessed by sampling the external ear canal with a cotton swab and transferring the material to a glass slide prior to heat fixing and staining. The criteria for demonstrating overgrowth were as described for the tape strips.

Culture of *Malassezia pachydermatis*

An isolate of *Malassezia pachydermatis* was obtained from the ear canal of a dog with *Malassezia* otitis. The use of a single isolate was deemed appropriate because previous studies have shown that *Malassezia pachydermatis* was the only *Malassezia* species isolated from the skin and external ear canal of dogs with either otitis externa or skin infections, and all isolates of *Malassezia pachydermatis* had similar electrophoretic karyotypes(29). The sample was cultured on Sabouraud Dextrose agar (Oxoid, UK) containing 20 mg/ml of chloramphenicol (Intramycetin®, Parke-Davis Veterinary, UK) for 48 h at 37°C. The colonies were then subcultured onto large numbers of plates in order to obtain enough organisms for subsequent studies.

Extraction of *Malassezia pachydermatis* proteins

Malassezia colonies carefully harvested and suspended in phosphate-buffered saline (PBS, pH 7.4) for a washing procedure that consisted of 3 cycles of centrifugation at 500 g for 5 min followed by removal of the supernatant and resuspension in PBS. After the last washing cycle, the cells were resuspended in extraction buffer (pH 7.4) containing 125 mM NH_4HCO_3 (Sigma, UK) and protease inhibitors (20 mM ε-aminocaproic acid, Sigma, UK; 5 mM ethylenediaminetetra-acetic acid, Sigma, UK; and 1 mM phenylmethylsulphonyl fluoride, BDH, UK). The *Malassezia* colonies in the extraction buffer were then mixed vigorously with an equal volume of glass beads (0.5 mm, 40 mesh, BDH, UK) on a vortex mixer for 10 min to mechanically disrupt the cell membranes. After extraction, the cell suspensions were stored at 4°C overnight, centrifuged at 6000 g for 5 min and the supernatants were collected. The amount of protein obtained was measured with BCA Protein Assay Reagent (Pierce Chemical Company, USA).

SDS-PAGE and electrophoretic transfer

Gel electrophoresis was performed according to the method of Laemmli(18) using 10% separating polyacrylamide gel and 4% stacking gel in a discontinuous buffer system containing 0.025 M Tris (Sigma, UK), 0.2 M Glycine (Fisher Scientific, UK) and 0.1% sodium dodecyl sulphate (Fisher Scientific, UK), pH 8.3. The extract (60 μg) and molecular weight standards were diluted 1 : 1 with reducing sample buffer (containing 5% β-Merca- ptoethanol, Sigma, UK) and heated at 95°C for 5 min. the extract was then added to one broad well across the top of the gel and the electrophoresis was run at 200 V for 40 min. Fig. 1 shows the protein profile of *Malassezia pachydermatis* extracts on a 10% separating polyacrylamide gel.

The separated proteins and molecular weight standards were transferred from the gel to a polyvinylidene difluoride microporous membrane (Millipore Immobilon™-P Transfer membrane, Millipore corporation, Bedford, MA, USA) in a Bio-Rad Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell according to the manufacturer's instructions. The transfer buffer contained 25 mM Tris (Sigma, UK), 192 mM Glycine (Fisher Scientific, UK), pH 8.3. The transfer was run at 80 mA per minigel for 1 h. The quality of transfer was checked by staining gels and molecular weight standards blotted onto the membrane with Coomassie Brilliant Blue R-250 (BDH, UK).

Intradermal testing

Aliquots of each extract were diluted in phosphate-buffered saline solution to protein concentrations of 20, 2, 0.2 mg/ml and 0.02 μg/ml. Before the skin tests, the hair was clipped from the lateral thorax with electric clippers with a number 40 blade. The dogs were injected intradermally with 0.05 ml of the extracts, using insulin syringes with 29 gauge needles

(Becton & Dickinson)USA, and with phosphate-buffered saline solution and histamine (1 : 100,000) as controls. Ten dogs were tested with all four dilutions of extracts. The test sites were inspected at intervals for evidence of hypersensitivity reactions, and the means of the horizontal and vertical diameters of wheals were recorded. The dogs were assessed after 15 minutes.

Statistical Analysis

The diameters of the reactions to the intradermal tests after 15 minutes in the healthy and affected dog were compared by analysis of variance, with analysis by student *t*-test. The analyses were made by using computer software (SigmaPlot 2000, Version 6.00). A significance level of $p < 0.05$ was used.

Results

The protcin profile of *Malassezia* pachydermatis extracts on a 10% separting polyacrylamide gel was shown in Fig 1. Malassezia pachydermatis protein extract showed several bands between 16 and 110 kDa in electrophoresis and the intensity was strondest between 25 to 80 kDa areas (Fig 1).

The lesions at the test sites of the 20 μg/ml concentrations of extracts in the affected dogs were significantly larger in diameter and thickness than in the healthy dogs. But the diameters of the lesions of other concentrations in the healthy and affected dogs were not significantly different.

Mean wheal diameter in the affected groups of 20, 2, 0.2, and 0.02 μg/ml was 13.36 ± 0.67, 5.33 ± 0.67, 5.47 ± 0.82, and 5.07 ± 0.64, respectively. Wheal diameter in the healthy groups of 20, 2, 0.2, and 0.02 μg/ml was 5.41 ± 0.48, 4.27 ± 0.28,

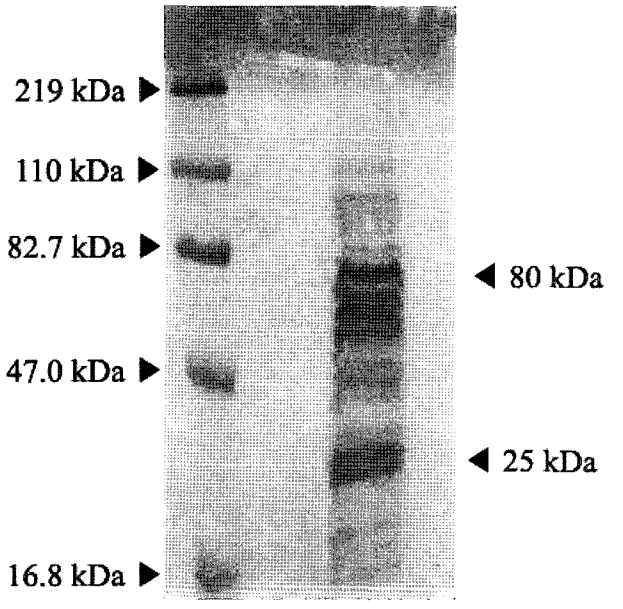

Fig 1. Coomassie Brilliant Blue stained *Malassezia pachydermatis* extracts on a 10% separating polyacrylamide gel. Lane 1: molecular weight markers; Lane 2: *Malassezia* extract.

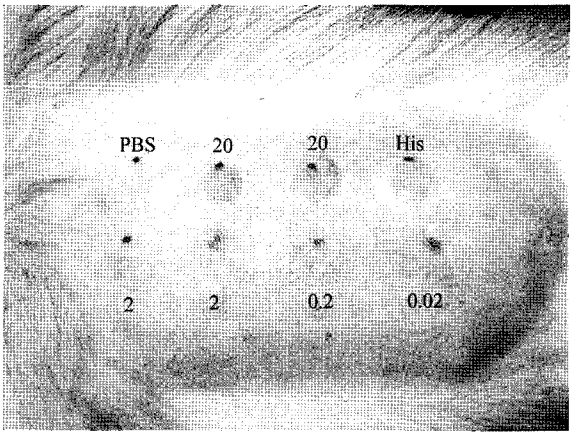

Fig 2. Intradermal reactions in the healthy dog 15 minutes after the injection of histamine, PBS control, 20 μg/ml 2 μg/ml, 0.2 μg/ml, 0.02 μg/ml. Large wheals are apparent at the site of injection of histamine, and a smaller wheal at the site of 20 μg/ml, 2 μg/ml, 0.2 μg/ml, 0.02 μg/ml, PBS control.

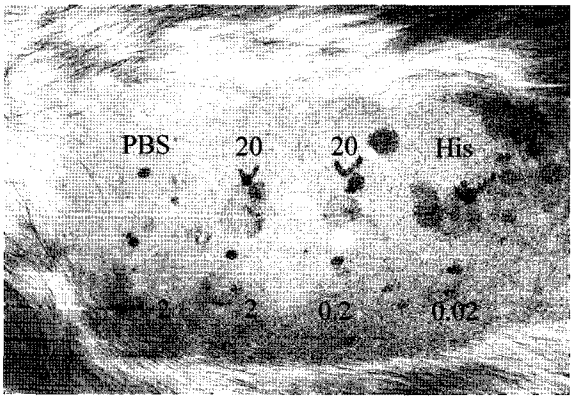

Fig 3. Intradermal reactions in the dog with *Malassezia* otitis 15 minutes after the injection of histamine, PBS control, 20 μg/ml, 2 μg/ml, 0.2 μg/ml, 0.02 μg/ml. Large wheals are apparent at the site of injection of histamine, 20 μg/ml, and a smaller wheal at the site of 2 μg/ml, 0.2 μg/ml, 0.02 μg/ml, PBS control.

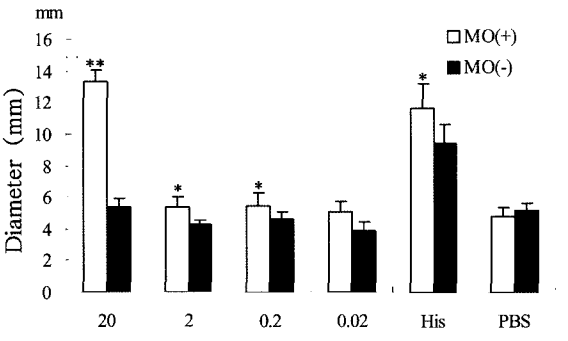

Fig 4. Wheal diameter of the intradermal wheal responses of healthy and affected dogs to a crude *Malassezia pachydermatis* extract at a concentration of 20, 2, 0.2, 0.02 μg/ml, histamine and PBS. (MO; *Malassezia* otitis, *p < 0.05, **p < 0.01)

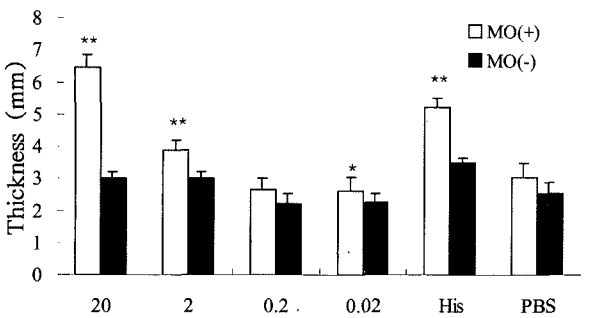

Fig 5. Wheal thickness of the intradermal wheal responses of healthy and affected dogs to a crude *Malassezia pachydermatis* extract at a concentration of 20, 2, 0.2, 0.02 μg/ml, histamine and PBS. (MO; *Malassezia* otitis, *p < 0.05, **p < 0.01)

4.61 ± 0.48, and 3.86 ± 0.52, respectively (Fig 4). The difference of wheal size between healthy and affected groups was significant.

Mean wheal thickness in the affected groups of 20, 2, 0.2, and 0.02 μg/ml was 6.44 ± 0.40, 3.86 ± 0.35, 2.64 ± 0.36, and 2.60 ± 0.44, respectively. Wheal thickness in the healthy groups of 20, 2, 0.2, and 0.02 μg/ml was 3.00 ± 0.19, 3.03 ± 0.20, 2.21 ± 0.32, and 2.24 ± 0.30, respectively (Fig 5). The difference of wheal thickness between healthy and affected groups was significant.

Discussion

The intradermal injection of substances derived from microbes and parasites may elicit a range of cutaneous inflammatory and hypersensitivity responses. Inflammation caused by irritant substances may develop without specific immunological sensitization; this may be the case with crude extracts derived from microbes (8). Antibody-dependent events include immunoglobulin E-mediated immediate (Type-I) and late-phase responses, and the Arthus reaction (Type-III hypersensitivity) (13).

In addition to testing healthy and affected dogs for immediate hypersensitivity responses to a crude extract of *Malassezia pachydermatis*, we desired to identify approximate molecular mass of any allergenic components of the yeast (14) by use of sodium dodecyl sulfate- polyacrylamide gel electrophoresis.

Malassezia yeasts are commonly associated with otitis externa in dogs and may contribute to sensitization (24). Our dogs had history of chronic or recurrent otitis externa. Five dogs in the *Malassezia* otitis-positive group reacted to the crude yeast extract, whereas the 5 dogs in the *Malassezia* otitis-negative group did not react. These results indicated that *Malassezia* otitis related to type-I hypersensitivity in dogs. However, further studies to evaluate the role and/or mechanisms of otic yeast in sensitized dogs will be required.

The observations confirm that *Malassezia pachydermatis*-derived antigens may induce an immediate wheal and flare response when injected intradermally in certain dogs (24). In this study, it seems reasonable to suggest that IgE-mediated hypersensitivity to yeast may contribute to the development of clinical signs in dogs with immediate skin test reactivity, especially in dogs with *Malassezia* otitis. Thus, The dogs' immediate skin test reactivity to *Malassezia pachydermatis* supports the idea that type I hypersensitivity responses may be important in the pathogenesis of *Malassezia* otitis. Also, it is possible that *Malassezia* otitis-dogs, through their extended pruritic behavior, are capable of becoming sensitized to their normal commensal populations of the yeast. It has been hypothesized that *Malassezia* allergen release may result from disruption of the organisms by the scratching behavior of the allergic individual or from digestion of the cell wall by enzymes released from inflammatory cells of affected skin (14). An additional contributing factor could be disruption of stratum corneum barrier function by scratching, allowing exposure of the sub-corneal immune system to yeast allergens.

The intradermal test did not discriminate between the healthy and affected basset hounds (4). But this difference may have been due to the use of antigens of different composition, their study was designed to investigate delayed reactivity or to the fact that the dogs tested were of different breeds and disease status.

In conclusion, the results of this study demonstrate that clinically healthy dogs will not manifest immediate hypersensitivity responses to intradermal injection of *Malassezia pachydermatis* extracts but that affected dogs with *Malassezia* otitis will manifest such hypersensitivity. However further studies are required to assess the relationship between the cutaneous responses to the intradermal injection of substances derived from *Malassezia pachydermatis* and the outcome of the colonization and infection of canine skin by the yeast. The variable immune responses observed among the dogs suggest that any future therapeutic or prophylactic immunomodulatory approach may need to target different processes mediated by the immune system of the skin.

Acknowledgements

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정상개와 *Malassezia* 외이염을 가진 개에 있어서 *Malassezia pachydermatis* 추출물의 즉시형 과민반응

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요 약 : *Malassezia pachydermatis* 추출물의 피내접종시 과민반응을 알아 보고자 본 실험을 수행하였다. 정상적인 개와 *M. pachydermatis* 외이염에 이환된 개를 대상으로 *M. pachydermatis* 추출물의 피내접종을 실시하였다. 전기영동을 통해 *M. pachydermatis* 분자량 조사를 통해 43~63 kDa을 포함하고 있는 추출물을 사용하였다. *Malassezia* 외이염에 감염된 군에서의 평균 팽진의 직경은 20, 2, 0.2, and 0.02 μg/ml 을 피내로 접종했을 때, 각각 13.36 ± 0.67, 5.33 ± 0.67, 5.47 ± 0.82와 5.07 ± 0.64을 나타내었으며, 팽진의 두께는 각각 20, 2, 0.2, and 0.02 μg/ml 에서 6.44 ± 0.40, 3.86 ± 0.35, 2.64 ± 0.36와 2.60 ± 0.44를 나타내었다. 통계학적으로 건강한 군과 외이염에 이환된 군에 있어서 유의한 차이가 인정되었다.($p < 0.01$). 더구나20 μg/ml 을 주입한 군에서 이 차이는 더욱 두드러져 농도 의존성을 나타내었다. 따라서 *M. pachydermatis* 유래 항원을 개의 피내에 접종했을 때, 즉시 팽진반응을 유발한다는 것이 확인되었으며 효모균에 대한 과민증은 즉시형 피부과민반응을 보이는 개, 특히 *Malassezia* otitis에 감염된 개에서 임상증상의 발현에 관여하는 것으로 사료된다. 또한 2, 0.2, 0.02 μg/ml 의 농도에서는 큰 차이를 보이지 않는 것으로 보아 즉시형 과민반응을 나타내기 위해서는 일정한 역치가 있을 것으로 생각되며 이에 대한 추가적인 연구가 필요할 것으로 사료된다.

주요어 : Malassezia pachydermatis, hypersensitivity, otitis externa, dogs.