

Evaluation of serum obtained from atopic dogs with dermatitis attributable to *Malassezia pachydermatis* for passive transfer of immediate hypersensitivity to that organism

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Objective—To determine the functionality of canine anti-*Malassezia* IgE via the passive transfer of immediate hypersensitivity localized to the skin (ie, cutaneous anaphylaxis) from atopic dogs with dermatitis attributable to overgrowth of *Malassezia pachydermatis* (*Malassezia* dermatitis [MD]) to healthy recipient dogs by use of the Prausnitz-Küstner (P-K) technique.

Animals—7 clinically normal dogs, 32 atopic dogs with MD, serum from 11 atopic dogs with MD, and 3 healthy dogs without prior sensitization to *M pachydermatis*.

Procedure—Serum from atopic dogs with MD was used for P-K tests in 3 clinically normal recipient dogs. Serial dilutions of untreated, heat-inactivated, IgE-absorbed, and bovine serum albumin (BSA)-absorbed (control) aliquots of serum were injected ID in triplicate for dermal sensitization. Twenty-four, 48, and 72 hours later, a crude extract of *M pachydermatis* was injected ID into the sites used for sensitization injections, and immediate hypersensitivity reactions were graded on a 4-point scale.

Results—Untreated serum caused P-K reactivity beginning 24 hours after passive sensitization and persisting through 72 hours (titers, 1:32 to 1:64). Heat inactivation and IgE-absorption of serum eliminated P-K reactivity, whereas treatment of serum with BSA did not.

Conclusions and Clinical Relevance—Analysis of P-K test results supports the passive transfer of cutaneous anaphylaxis by anti-*Malassezia* IgE and indicates it is functional in type-1 hypersensitivity reactions of atopic dogs with MD. Reduction or blockade of anti-*Malassezia* IgE in atopic dogs with MD may provide better clinical control of the disease. (*Am J Vet Res* 2003;64:262–266)

Malassezia pachydermatis (formerly *Pityrosporum canis*) is a lipophilic yeast that colonizes the skin

of healthy dogs in low numbers.¹ However, it is also an opportunistic pathogen that may flourish on the skin of dogs when the barrier function of the stratum corneum is compromised or when there is increased lipid content of the skin surface (seborrhea). Excessive colonization of the skin by *M pachydermatis* (ie, *Malassezia* dermatitis [MD]) is often associated with marked inflammation and pruritus.^{1–3} Relative numbers of the organism that are expected to be found on the skin of healthy dogs and the inflamed skin of dogs with atopic dermatitis (AD) have been determined by use of cytologic techniques.^{4,5}

The host immune response to *M pachydermatis* has been characterized in dogs with AD. Dogs with an AD phenotype have type-1 hypersensitivity reactions to ID injection of a crude extract of *M pachydermatis*.^{5,6} Atopic dogs also have increased serum concentrations of anti-*Malassezia* IgE as determined by use of an ELISA technique⁷ and an increase in lymphocyte blastogenesis in vitro in response to administration of a crude extract of *M pachydermatis*.⁶ Therefore, it may be concluded that *M pachydermatis* is recognized by the immune system of dogs in a manner similar to that for aeroallergens. The same is true for atopic human beings in which a similar yeast, *M furfur*, contributes to the pathologic consequences of AD.⁸ In contrast, any relationship between hypersensitivity to *Malassezia* organisms and concurrent dietary sensitivity is only speculative and has not been investigated to the knowledge of the authors.

Despite in vivo documentation of immediate hypersensitivity reactions to *Malassezia* extracts in some patients and in vitro documentation of *Malassezia*-specific IgE, the link between these 2 findings (ie, functionality of serum anti-*Malassezia* IgE to cause immediate hypersensitivity reactions) has not been determined. The true test of this functionality would be the use of serum to transfer type-1 reactivity from an affected dog to a normal recipient dog. This can be achieved by use of the Prausnitz-Küstner (P-K) test or the passive cutaneous anaphylaxis (PCA) test. The shared purpose of these testing modalities is to transfer specific IgE antibody from the serum of a donor to the dermal mast cells of a nonsensitized recipient, which will then function in an immediate hypersensitivity reaction when exposed to the specific antigen. The term cutaneous anaphylaxis is used to describe the allergen-induced degranulation of mast cells with release of proinflammatory mediators, which is localized to the skin. To achieve sensitization for the P-K or PCA tests, serum obtained from a donor is

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injected ID into a recipient. However, in the P-K test, the specific antigen is also injected ID at sites used for sensitization, whereas in the PCA test, the allergen is administered IV. The P-K test is often preferred to the PCA test, because the latter is more technically demanding, more time consuming, and may be less sensitive.⁹ Because the challenge-exposure dose for IV administration has not been standardized, false-negative reactions are possible when an inadequate amount of allergen reaches the skin.¹⁰

Because of differences in the duration of antibody retention within the skin of a recipient, the P-K and PCA tests allow investigators to discriminate IgE-mediated reactions from possible non-IgE anaphylactic antibody-mediated reactions, such as those that may result with IgG subclasses.¹¹ The objective of the study reported here was to determine the functionality of canine anti-*Malassezia* IgE by transfer of immediate hypersensitivity localized to the skin (ie, cutaneous anaphylaxis) from atopic dogs with MD to healthy recipient dogs by use of the P-K technique.

Materials and Methods

Animals—Several dogs were used in the 3 experiments for the study reported here. In experiment 1, 7 clinically normal dogs were used in an allergenic threshold study. In experiment 2, 32 client-owned atopic dogs were evaluated by use of intradermal tests (IDTs). In experiment 3, serum from atopic dogs with MD was injected into 3 healthy recipients during P-K testing. All in vivo experiments were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Experiment 1—Seven clinically normal dogs were used in an allergenic threshold study to determine the concentration of crude extract of *M pachydermatis* most appropriate for use in an IDT. Dogs were considered clinically normal on the basis of results of physical and dermatologic examinations. None of the dogs had received any type of glucocorticoid medication for a minimum of 30 days or any antihistaminic medication for a minimum of 14 days before onset of the study. The dogs lacked cytologic evidence for MD (defined as ≤ 3 *Malassezia* organisms/0.6 cm² of an area on a microscopic glass slide containing a direct skin impression, as reported elsewhere.⁴)

To develop a crude extract for threshold testing, yeast organisms were isolated from a dog with MD by swabbing the skin with a sterile cotton-tipped applicator and transferring the material to plain Sabouraud's dextrose agar. Cultures were incubated at 37°C for 6 days, and individual colonies were identified as *Malassezia* spp by direct microscopic examination. A crude extract of the colonies was prepared by use of a vortex cell homogenizer with microglass beads^a in accordance with techniques reported elsewhere.⁵

The crude extract was lyophilized and weighed. Protein content of the extract was estimated by use of a commercial colorimetric assay.^b Lyophilized material was reconstituted with 0.05M PBS solution (pH, 7.2) to achieve a stock concentration of 20,000 µg/mL (dry weight) and stored at -80°C in 1-mL aliquots. The stock concentration and dilutions (concentrations of 2,000, 200, 20, and 2 µg/mL) were used for the skin testing threshold study.

Experiment 2—Thirty-two client-owned dogs that were admitted for evaluation of pruritic dermatitis at the dermatology service of each of 2 veterinary medical teaching hospitals were used in the second experiment. All dogs were sus-

pected of being atopic on the basis of appropriate signalment, history, clinical signs at the time of admission,¹² and a reaction score of $\geq 2+$ for at least 3 of the commercial aeroallergens used in a standard IDT protocol. Concurrent dietary sensitivity was not ruled out in these dogs, because it was not considered germane to the study. All dogs met the aforementioned medication withdrawal times and were not bathed for ≥ 14 days prior to evaluation.

Samples (skin impressions and ear exudate) were collected from each of the 32 atopic dogs and evaluated cytologically for MD and for otitis attributable to an overgrowth of *M pachydermatis* (*Malassezia* otitis [MO]). Dogs were grouped on the basis of results of an IDT and cytologic evaluation into 3 categories: atopic dogs with MD, atopic dogs with MO, and atopic dogs without MD or MO (atopic control dogs). Lack of MD was determined by the same cytologic criteria used for the clinically normal dogs without atopy in experiment 1. Diagnosis of MD was determined by finding ≥ 10 yeast organisms/0.6 cm² of area on a glass microscope slide.⁷ A positive result on slides for ≥ 1 sample site was required for inclusion as an atopic dog with MD. *Malassezia* otitis was defined as > 4 yeast organisms/hpf on slides containing exudate obtained from the ear canal.¹³

To avoid investigator bias when evaluating IDT reactions, dogs were assigned to each of the 3 atopic groups after all other testing procedures were concluded. Five milliliters of blood was collected from each dog into a serum separator tube. Serum was stored at -80°C until needed for in vitro studies.

ELISA testing—Sera from the 32 atopic dogs and 6 of 7 healthy dogs were evaluated to determine content of anti-*Malassezia* IgE by use of an ELISA method. Serum from the other healthy dog was lost during transport to the laboratory. Briefly, polystyrene microtiter plates were coated with a crude extract of *M pachydermatis* (100 µL of a solution of 500 µg/mL) and allowed to incubate overnight at 4°C. Wells were blocked with 0.4% bovine serum albumin (BSA) solution by incubation for 2 hours at 20 to 23°C; all remaining steps were performed at 20 to 23°C (ie, room temperature). Serum samples were added in duplicate to wells at a dilution of 1:5, and the plates were incubated for 4 hours. Monoclonal mouse anti-canine IgE¹⁴ (3 µg/mL) was then added to the wells, and plates were incubated for 2 hours. Finally, alkaline phosphatase-conjugated donkey anti-mouse IgG^c (dilution, 1:1,000) was added to all wells, and plates were incubated for 1 hour. Color in each well was developed by the addition of *p*-nitrophenyl phosphate substrate solution, and color development was monitored by use of a microplate reader until an internal positive-reference sample on each plate reached an optical density (OD) of approximately 1.000. Values for blank wells that contained only substrate were automatically subtracted from the value for each experimental well. Preliminary experiments with sera from atopic dogs revealed that a few serum samples had high background binding (OD > 0.150) in this assay (data not shown). Therefore, each sample was tested with the anti-IgE reagent included in the assay protocol and again with the anti-IgE reagent deleted and replaced by buffer; this created an individual serum background control value for each sample. The OD of each well on a plate was adjusted on the basis of the positive-reference sample for that plate by dividing the sample ODs by the value for the positive-reference value. Each individual serum background control value was subtracted from results for each sample, and the net result was reported as the mean adjusted OD value.

Experiment 3—We conducted P-K tests in 3 healthy recipient dogs. Two serum sources that could cause potential reactivity to *Malassezia* spp were selected for evaluation. Serum 1 consisted of pooled (equal volumes) sera from 10 of

the atopic dogs with MD. These 10 sera were selected on the basis that each of these dogs had a strong (3+ to 4+) reaction to *M pachydermatis* antigen during the IDT, regardless of the ELISA values. However, 1 of these 10 sera did have a moderately high OD for anti-*Malassezia* IgE on the ELISA. Serum 2 consisted of a large volume of serum from a single dog. This serum had high concentrations of anti-*Malassezia* IgE when tested by use of the ELISA. Serum 2 was obtained from an atopic dog with MD that was identified independently by 1 of the investigators (DJD), but it was not included in the atopic study and not available for an IDT.

Serum 1 was evaluated in the P-K test by use of 2 conditions (untreated serum and serum that underwent heat inactivation to destroy IgE reactivity). Serum 2 was evaluated by use of 4 conditions (untreated serum, serum that underwent heat inactivation to destroy IgE reactivity, serum after removal of IgE by use of immunoabsorption with anti-IgE coated beads, and serum after treatment with BSA-coated beads [immunoabsorption control sample]). For heat inactivation, aliquots of serum 1 and 2 were heated in a water bath (56°C) for 4 hours. For immunoabsorption, 5 mg of monoclonal mouse anti-IgE antibody was covalently coupled to 1 mL of carbonyldiimidazole-activated agarose beads in accordance with the manufacturer's protocol.⁴ Beads were washed extensively in PBS solution and pelleted by a brief centrifugation. A 5-mL aliquot of serum 2 was added to the beads, and the mixture was incubated for 18 hours at 20 to 23°C with gentle agitation. The beads were allowed to settle by gravity, and absorbed serum supernatant was recovered and stored frozen until used in the P-K test. For the control immunoabsorption, agarose beads were coupled to 5 mg of BSA, and another 5-mL aliquot of serum 2 was treated with the BSA-coated beads as described previously.

To perform the P-K test, aliquots of test sera (untreated, heated, absorbed, or absorption-control) were diluted with PBS solution in five 2-fold serial dilutions (1:2 to 1:64). Three healthy dogs that did not have skin abnormalities or cytologic evidence of MD were used as recipients. Because of the large number of injections required, experiments were performed during a 3-month period such that there was a 4-week interval between tests. Untreated serum was always tested in parallel with 1 or more of the treated sera. For each dilution of each sera, 0.05 mL was injected ID (in triplicate) in 3 rows on the lateral thorax of the recipient dogs. The PBS solution was used as a negative-control injection, histamine phosphate was used as a positive-control injection, and the crude extract of *M pachydermatis* (20 µg/mL) was used to rule out prior sensitization in the test subjects. Twenty-four, 48, and 72 hours after ID injections, a volume of the crude extract of *M pachydermatis* was injected into the exact sites used for the serum injections (1 row used for each time point). Reactions were graded on a scale of 0 to 4+ on the basis of comparison with the reactions for the PBS solution (0) and histamine phosphate (4+) injections. The crude extract of *M pachydermatis* was again injected at each time point as a control measure to rule out sensitization from repeated injection during prior experiments. All reactions were graded at 15 minutes, 4 hours, and 24 hours after antigen injection. The P-K titer of a sample was defined as the highest dilution that resulted in an immediate positive reaction of ≥ 2+.

Statistical analysis—The Kruskal-Wallis test was used for statistical comparisons of ELISA results among groups. For all comparisons, a value of $P < 0.05$ was considered to indicate a significant difference. All statistical evaluations were performed by use of a statistical software package.⁶

Results

The *M pachydermatis* antigen contained 6% protein on a dry-matter basis. The allergenic threshold of

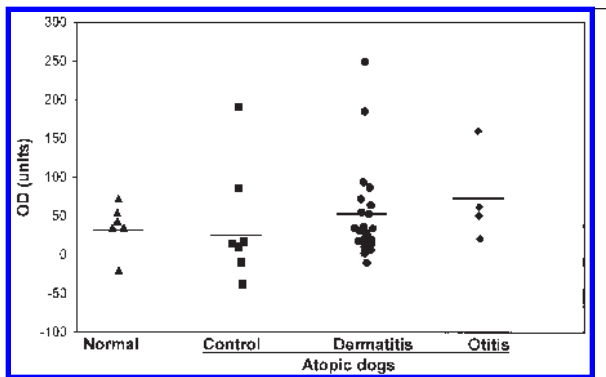


Figure 1—Amounts of anti-*Malassezia* IgE in serum samples obtained from clinically normal dogs (normal) and 3 groups of atopic dogs (atopic dogs without evidence of dermatitis or otitis attributable to overgrowth of *Malassezia pachydermatis* [control], atopic dogs with evidence of dermatitis attributable to overgrowth of *M pachydermatis* [dermatitis], and atopic dogs with evidence of otitis attributable to overgrowth of *M pachydermatis* [otitis]) on the basis of optical density (OD) scores determined by use of an ELISA. Each symbol represents the value for 1 dog. Horizontal bars represent mean values for each group of dogs.

the extract used for the IDT was 2,000 µg/mL, because 6 of 7 clinically normal dogs reacted to the stock concentration of 20,000 µg/mL, but none of the dogs reacted to an injection at a dilution of 2,000 µg/mL.

The 32 atopic dogs were classified into 3 groups (21 dogs with MD, 4 dogs with MO, and 7 atopic control dogs). Data for the ELISA were not normally distributed. Optical density values were determined for dogs in each of the 3 atopic groups from experiment 2 and for 6 of the 7 clinically normal dogs from experiment 1 (Fig 1). Although several dogs had low to moderate OD values for *Malassezia*-specific IgE, we did not detect significant differences in mean ELISA values among atopic groups or between any atopic group and the group of clinically normal dogs.

The 3 recipient dogs had positive results during the P-K test with untreated sera (ie, untreated serum 1 and untreated serum 2). Wheal-and-flare reactions were evident within 15 minutes after extract injection. Reactivity was evident at 24 hours after passive sensitization and persisted at the 72-hour evaluation. We did not detect delayed reactions at 4 or 24 hours after extract injection.

Aliquots of both untreated test sera had titers of 1:32 to 1:64. Reactions were scored 4+ for the 1:32 dilution, but decreased to 1+ or 2+ (depending on the recipient dog) for the 1:64 dilution. Heat treatment completely abrogated reactivity of both sera. The aliquot of serum 2 absorbed with anti-canine IgE was nonreactive in all recipients; however, the immunoabsorption control serum (treated with BSA-coupled beads) maintained reactivity with a titer of 1:32 in all 3 recipient dogs. None of the dogs reacted to control *M pachydermatis* injections at any time during the study, indicating that recipient dogs did not develop active sensitization as a result of multiple ID injections of the allergen during the 3-month period.

Discussion

Analysis of results of the study reported here indicates that atopic dogs with MD and evidence of imme-

diate-type hypersensitivity to *Malassezia* antigen, as determined on the basis of results of an IDT or ELISA, can transfer this reactivity to recipient dogs in a P-K test. Passive transfer of such reactivity with serum is direct evidence for the functional reaginic antibody in the donor dogs from which serum was obtained. Moreover, persistence of reactivity for at least 72 hours in the recipient dogs and elimination of this reactivity by use of heat-inactivated or IgE-absorbed sera are evidence that the responsible reaginic antibodies are, at least in part, of the IgE class.^{15,16}

Although most of the atopic dogs with MD tested in the study had positive results when injected with the *Malassezia* antigen during the IDT, we found few of these dogs to have substantial amounts of anti-*Malassezia* IgE when tested by use of the ELISA. Discrepancies between in vitro assays for allergen-specific IgE and positive reactions during an IDT with the same allergen are common in clinical practice and often explained by the possibility that most of the IgE is localized in the skin rather than the circulation.¹⁷ We were unable to document a significant difference in reactivity between atopic dogs with MD and clinically normal dogs on the basis of values for the ELISA. This result is in contrast with results of another study⁷ in which investigators reported frequent detection of *Malassezia*-specific IgG and IgE in sera from atopic dogs, with concentrations of these specific antibodies significantly higher than those in clinically normal control dogs. In another report,¹⁸ investigators identified several major allergens of *M. pachydermatis* via use of western immunoblotting to which more than half of the dogs were reactive. Numerous technical differences in the methods of preparation of the *Malassezia* extract, assay reagents used, and ELISA methods used in the study reported and in those other studies may explain the differing results. Another consideration is heterogeneity of IgE, which has been documented in human beings and dogs via numerous techniques. In the sentinel study¹⁹ of dogs, 2 subforms of canine IgE were identified and defined on the basis of differential charge, affinity to protein A, and antigenicity. One of these subforms did not react with a monoclonal anti-canine IgE antibody in an ELISA, whereas the other subform was reactive. However, both subforms were reactive in a PCA test and an ELISA that used a polyclonal anti-canine IgE. In another study,⁹ the coefficient of correlation between ELISA and PCA results for anti-ragweed IgE in dogs was higher when investigators used a polyclonal anti-IgE antibody than when they used a monoclonal antibody. Because the ELISA in the study reported here used a monoclonal anti-canine IgE antibody, it is possible that the monoclonal antibody did not recognize the *Malassezia*-specific IgE that was responsible for our in vivo positive reactions during IDT and P-K testing.

Some atopic dogs without evidence of MD or MO by our cytologic definition, as well as some clinically normal dogs, had low amounts of anti-*Malassezia* IgE that were not significantly different from the concentrations in the dogs of the atopic groups, and 1 clinically normal dog had a moderately high concentration (Fig 1). Low to moderate amounts of circulating anti-

body in such dogs may have been the result of prior episodes of MD or MO that were inactive at the time of sample collection, or they could have represented a normal immune response to an organism that is found in all healthy dogs as part of the commensal microflora. Assuming the latter to be true, the phenomenon may not be dissimilar from the reactions many clinically normal dogs have to common environmental allergens such as house dust mites.²⁰

Similar to atopic human beings, dogs may express non-IgE-sensitizing antibodies (IgGd in dogs; IgG4 and IgG1 in human beings) targeted at specific allergens.^{11,21} Although the importance of these antibodies in atopic disease in human beings and dogs has been questioned,^{20,22} some experimental evidence supports a role for IgG4 as an anaphylactic antibody in human beings.²² If allergen-specific IgGd plays an etiologic role in hypersensitivity to *Malassezia* organisms in dogs, IgE-specific ELISAs would underestimate the total amount of circulating antigen-specific anaphylactic antibody, whereas the IDT or P-K test might be reactive regardless of the antibody isotype bound to mast cells in the skin. However, results of the P-K test in the study reported here were strongly supportive of the passive transfer of anti-*Malassezia* IgE, rather than an IgG subclass, from atopic dogs to healthy recipient dogs.

In most species, the optimal sensitization period for IgG is 3 to 4 hours, and reactivity dissipates by 24 hours,^{15,16} whereas the optimal sensitization period for IgE is 24 to 48 hours, and reactions may persist for several days.¹⁵ Reactivity to both untreated sera in the study reported here persisted until the evaluation at 72 hours after injection. In addition, IgE, but not IgG, is heat-labile after incubation at 56°C for 4 hours. Heating of the 2 test sera completely eliminated reactivity during the P-K test. Finally, immunoabsorption of serum 2 with monoclonal mouse anti-canine IgE also completely eliminated reactivity during the P-K test, whereas the aliquot treated with BSA (which should not have greatly affected the serum content of IgE) did not abrogate reactivity during the P-K test. Again, these results indicated that the immediate hypersensitivity reactions were IgE-specific.

Analysis of results of the P-K test supports the passive transfer of cutaneous anaphylaxis by anti-*Malassezia* IgE. Furthermore, analysis indicates that anti-*Malassezia* IgE functions in type-1 hypersensitivity of atopic dogs with MD.

^aBead Beater, Biospec Products Inc, Bartlesville, Okla.

^bCoomassie Plus protein assay, Pierce Chemical Co, Rockford, Ill.

^cDonkey anti-mouse IgG, Jackson ImmunoResearch Laboratories Inc, West Grove, Pa.

^dReacti-Gel 6X, Pierce Chemical Co, Rockford, Ill.

^eIntercooled Stata 7.0 for Windows 98/95/NT, Stata Corp, College Station, Tex.

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