Response to *Malassezia pachydermatis* by peripheral blood mononuclear cells from clinically normal and atopic dogs

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**Objective**—To investigate the potential cell-mediated immune response of atopic dogs to the yeast *Malassezia pachydermatis* and to correlate it with the type-1 hypersensitivity (humoral) response of the same population of dogs.

**Animals**—16 clinically normal dogs, 15 atopic dogs with *Malassezia dermatitis*, 5 atopic dogs with *Malassezia otitis*, and 7 atopic control (ie, without *Malassezia dermatitis* or otitis) dogs.

**Procedure**—A crude extract of *M. pachydermatis* was extracted for use as an intradermal allergy testing reagent and for stimulation of isolated peripheral blood mononuclear cells in vitro. Flow cytometry was also used to assess cell surface antigenic determinants (CD3, CD4, CD8, CD14, CD21, CD45RA, surface immunoglobulin) on peripheral blood mononuclear cells.

**Results**—Atopic dogs with cytologic evidence of *Malassezia dermatitis* had an increased lymphocyte blastogenic response to crude *M. pachydermatis* extract, compared with clinically normal dogs and dogs with *Malassezia otitis*. Atopic control dogs did not differ significantly in their responses from atopic dogs with *Malassezia dermatitis* or otitis. A significant correlation was not found between the lymphocyte blastogenic response and the type-1 hypersensitivity response to *M. pachydermatis* within any of the groups.

**Conclusions and Clinical Relevance**—Cell-mediated and humoral reactivities to *M. pachydermatis* contribute to the pathogenesis of atopic dermatitis in dogs but are not directly correlated. Modification of the dysregulated immune response toward *M. pachydermatis* may assist in the reduction of pathologic changes associated with an atopic dermatitis phenotype in dogs. *(Am J Vet Res 2002;63:358–362)*

*Malassezia pachydermatis* (formerly *Pityrosporum canis*), a nonmycelial unipolar lipophilic budding yeast, is a known commensal organism as well as a pathogen of canine skin. Although the organism is easily cultured from the noninflamed skin of clinically normal dogs, it is quite difficult to identify by cytologic techniques (direct impression smear, cotton-tip swab, dry skin scrapings) from the same dogs.

However, when found in high numbers from the skin of inflamed or seborrheic skin, the term *Malassezia dermatitis* (MD) is applied. The distinct and predictable response to antifungal chemotherapy, which ameliorates much of the pruritus and erythema associated with MD, must often be repeated for recurrent infections in predisposed dogs. Because of potential toxicity associated with such treatment, other methods of treatment or prophylaxis are needed.

Clinical associations between an atopic dermatitis (AD) phenotype and MD in dogs have been confirmed by the report of type-1 hypersensitivity reactions to intradermally injected crude extracts of *M. pachydermatis*. Atopic dogs with cytologically confirmed MD had increased reactivity to the yeast, compared with atopic dogs without MD. Therefore, it was concluded that *M. pachydermatis* is recognized by the immune system of dogs in a manner similar to aeroallergens. However, the cell-mediated immune response of atopic dogs to the yeast has not been previously reported. The cell-mediated immune response is an important component of the paradigm for atopy in humans, which proposes that the immunologic basis of AD is T-lymphocyte (T-cell) dysregulation.

The purposes of the study reported here were to investigate the cell-mediated immune response of dogs to *M. pachydermatis* and to compare it with the concurrent humoral reactivity. We planned to test the hypothesis that peripheral blood mononuclear cells (PBMC) of atopic dogs with MD would have increased lymphocyte blastogenesis in response to a crude extract of *M. pachydermatis*, compared with PBMC of clinically normal dogs. However, on the basis of the results of a prior study that evaluated humoral reactivity to the yeast, we hypothesized that the PBMC response of atopic dogs without MD would not differ substantially from that of clinically normal dogs.

**Materials and Methods**

**Extraction of organisms**—Yeast organisms were harvested from the skin of a dog with MD by swabbing the skin with a sterile cotton-tip applicator and transferring the material to plain Sabouraud dextrose agar. Organisms were incubated at 37°C for 6 days, and individual colonies were identified as *Malassezia* sp by direct microscopic examination. Extraction was performed with a vortex cell homogenizer with microglass beads, using the techniques reported previously.

**Antigen preparation**—The crude extract was lyophilized and weighed. The protein concentration on a dry matter basis was estimated by Bradford technique. The lyophilized material was reconstituted with PBS solution to a concentration of 20 mg/ml and stored at –80°C in 1-ml aliquots. For the skin-
testing threshold study, the stock concentration was used and was also further diluted to concentrations of 2,000 µg/ml, 200 µg/ml, 20 µg/ml, and 2 µg/ml for injection. For lymphocyte blastogenesis assays, the extract was filtered through a syringe-top filter with a pore size of 0.45 µm and was diluted to 250 µg/ml, 125 µg/ml, 62 µg/ml, and 31 µg/ml.

In vivo studies: phase 1 and 2—All in vivo experiments were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Seven clinically normal dogs were used in a threshold study (phase 1) to determine the concentration of M. pachydermatis crude extract most appropriate for intradermal allergy testing (IDAT). On the basis of these findings, 27 atopic dogs were evaluated by IDAT (phase 2).

Phase 1: allergenic threshold study—Seven dogs that were determined to be clinically normal on the basis of the results of complete physical and dermatologic examinations were used in an allergenic threshold study. These dogs had not received any steroidal medication orally, parenterally, or topically for a minimum of 30 days nor any antihistaminic medication for a minimum of 14 days. These dogs lacked a direct skin impression as previously reported or for Malassezia pachydermatis (MO; defined as <4 yeast organism/100X oil immersion field of exudate from the ear canal).

Dogs were intradermally tested with the 60 commercial aerogelallergens used in our teaching hospital for the evaluation of pet dogs with a clinical diagnosis of AD as well as the 6 dilutions of the M. pachydermatis extract. Controls and grading were performed as previously reported. A wheal-and-flare reaction ≥2+ was considered clinically relevant. Dogs reacting to >1 of the commercial aerogelallergens were disqualified. The threshold concentration of Malassezia crude extract appropriate for skin testing of atopic dogs was defined as the maximum concentration to which clinically normal dogs without atopy ceased to react.

Phase 2: testing of atopic dogs—Twenty-seven privately owned dogs that were admitted to the dermatology service for pruritic dermatitis were evaluated. All dogs were suspected of being atopic on the basis of appropriate signalment, history, clinical signs at the time of admission, and a ≥2+ reaction to ≥3 of the commercial aerogelallergens used in our standard skin testing protocol. All dogs satisfied standardized medication withdrawal times established by our dermatology service and could not have been bathed for 14 days prior to sample collections.

The 27 atopic dogs were grouped as follows on the basis of the results of IDAT and cytologic skin impression and ear exudate: dogs with MD (atopic MD dogs), atopic dogs with only MO (atopic MO dogs), and atopic dogs with neither MD nor MO (atopic control dogs). Absence of MD was determined by the same cytologic criteria used for the clinically normal dogs without atopy in phase 1. Presence of MD was determined by the finding of ≥10 yeast organisms/0.25 in² area of microscopic glass slide. A positive slide from ≥1 sampling sites was required for inclusion with the atopic MD dogs. Group assignments were performed after all other testing procedures were concluded to avoid investigator bias in evaluating IDAT reactions.

Lymphocyte blastogenesis assays—Peripheral blood mononuclear cells were collected from the 7 clinically normal dogs and an additional 9 clinically normal dogs that were not included in the threshold study (but that were subjected to the same criterion for normalcy). Peripheral blood mononuclear cells were also collected from the 27 atopic dogs. From each of the 16 clinically normal and 27 atopic dogs, 10 ml of whole blood was collected into EDTA-treated tubes for PBMC isolation. Mononuclear cells were isolated from samples by hypaque-Ficoll density gradient, and 2 x 10⁶ cells suspended in 200 µl medium/well were incubated for 7 days in 96-well flat bottom plates with M. pachydermatis extract added at concentrations of 250 µg/ml, 125 µg/ml, 62 µg/ml, and 31 µg/ml. Six samples at each concentration and 12 control samples (6 negative controls consisting of cell medium only and 6 positive controls to which phytohemagglutinin was added) were run from each dog. After 6 days, 0.5 µCi of [³H] thymidine was added to each well for the remaining 18 to 24 hours of culture. Cells were harvested onto glass fiber filters, and thymidine incorporation was determined by liquid scintillation spectrometric counting and expressed as cpm. The concentration of M. pachydermatis extract that achieved maximum stimulation of cells was used for determination of the stimulation index (SI), using the equation:

\[ \text{SI} = \frac{\text{mean cpm of PBMC + M. pachydermatis (62 µg/ml)}}{\text{mean cpm of PBMC + medium alone}} \]

Flow cytometry—The PBMC were isolated and stained for flow cytometric analysis. Negative control samples were stained only with secondary antibody. For each sample, worn 10,000 cells were analyzed, using an automated cell counter. The murine monoclonal antibodies used in this study included CA17.2A12 (canine CD3), CA2.1D6 (canine CD21), and CA4.1D3 (canine CD45RA). A fluorescein isothiocyanate (FITC)-labeled canine surface immunoglobulin was purchased. Secondary antibodies used included anti-mouse FITC-labeled IgG or anti-mouse phycoerythrin (PE)-labeled IgG. The rat monoclonal antibodies used were FITC-labeled CD4 and PE-labeled CD8. As isotype controls, rat PE-labeled IgG2a and rat FITC-labeled IgG1 were used for the directly labeled rat antibodies.

Statistical analysis—Nonparametric tests were used for statistical comparisons of surface antigen expression and SI among groups. The Kruskal-Wallis test was initially used to detect any differences among groups. If results of the Kruskal-Wallis test were significant, the Wilcoxon rank-sum test was used for pairwise comparisons. The Kappa statistic was used to assess the amount of agreement between a positive or negative SI result and a positive or negative IDAT result. For all statistical comparisons, a P value of <0.05 was considered significant. All statistical evaluations were performed, using a statistical software package.

Results

The M. pachydermatis antigen that was prepared was composed of 6% protein on a dry matter basis. The allergenic threshold of the extract used for IDAT was 2,000 µg/ml; whereas 6 of 7 clinically normal dogs reacted to the stock concentration of 20 mg/ml, none of them reacted to the 2,000 µg/ml dilution.

The 27 atopic dogs were grouped as follows: 15 atopic MD dogs, 5 atopic MO dogs, and 7 atopic control dogs. The IDAT results for atopic dogs proved an overall 74% positive response rate to the M. pachydermatis extracts, when ≥2+ wheal-and-flare responses were considered. Twenty of the 27 atopic dogs reacted to the threshold concentration of the crude extract, which included 13 of 15 MD dogs, 4 of 5 MO dogs, and 3 of 7 control dogs. Differences were not significant between the various groups of atopic dogs (MD vs MO dogs, P = 0.982; MO vs control dogs, P = 0.293; control vs MD dogs, P = 0.054).

Data from the lymphocyte transformation assay were not normally distributed (Fig 1). Defining positive SI results as ≥2.5 revealed significant differences...
among groups (P = 0.035). Using pairwise comparisons, atopic MD dogs had significantly greater median SI than clinically normal dogs (P = 0.022) and atopic MO dogs (P = 0.044). However, a significant difference was not found in the median SI between other groups (atopic MD vs atopic control dogs, P = 0.860; atopic control vs atopic MO dogs, P = 0.088; and atopic MO dogs vs clinically normal dogs, P = 0.709). The degree of agreement between a positive or negative SI result, and a positive or negative IDAT result was poor. Test results were in agreement 40.74% of the time, which was not significant (P = 0.886).

Significant differences were not found in surface antigen expression between PBMC of clinically normal dogs and any atopic group dogs except for the ratio of CD4+:CD8+ cells in atopic MD dogs. Atopic MD dogs had a significantly increased CD4:CD8 ratio (mean, 3.2; range 1.2 to 6.0, SD ± 1.0), compared with clinically normal dogs (mean, 2.0; range 1.0 to 2.8, SD ± 0.6; P = 0.021). Ratios for the atopic MO (mean, 2.7) and atopic control (mean, 2.2) dogs were higher than for the clinically normal dogs, but these differences were not significant.

### Discussion

In humans with AD, hypersensitivity to M furfur (formerly Pityrosporum ovale and P orthiculum) has been confirmed by several independent groups, using IDAT to as well as skin prick testing. Moreover, the atopy patch test, and in vitro identification of anti-M furfur IgE by ELISA and radio allergosorbent test techniques. Furthermore, the cell-mediated immune response to M furfur has also been investigated for humans with AD; the PBMC response and the in situ lymphocyte response to yeast extracts for humans have been characterized in

Figure 1—Box plots of the stimulation indices (SI) of the peripheral blood mononuclear cells (PBMC) from atopic dogs with Malassezia dermatitis (affected), atopic dogs with Malassezia otitis (otitis), clinically normal dogs (normal), and atopic dogs with neither Malassezia dermatitis nor Malassezia otitis (control). The PBMC were incubated with M pachydermatis extract (62 µg/ml). Lower limits of the line extensions represent the 10th percentile. The boxes extend from the 25th to 75th percentiles. Solid lines within boxes represent medians. Open circles are outliers. Significant differences (P < 0.05) were detected between PBMC from atopic dogs with Malassezia dermatitis versus clinically normal dogs and between PBMC from atopic dogs with Malassezia dermatitis versus atopic dogs with Malassezia otitis. Numbers in parenthesis represent the number of dogs in each group.
with atopy has been a topic of controversy, we wanted to evaluate the potential effect of MD on T-cell kinetics. In our atopic MD dogs, the CD4:CD8 ratio was significantly increased (as assessed by flow cytometric analysis of PBMC), compared with clinically normal dogs, but this did not hold true for atopic control and MO dogs. The ratio in our clinically normal dogs (2.0) was similar to that reported previously for clinically normal dogs, cats, and humans. Comparison of PBMC from humans with AD to PBMC from clinically normal individuals has resulted in conflicting data; increased and normal CD4:CD8 ratios have resulted, whereas in cats a difference was not found between PBMC from clinically normal and atopic cats. In cats, dogs, and humans with AD, the lymphocytic infiltrate of the skin consists predominantly of T cells that have a consistently higher CD4:CD8 ratio than that of skin from clinically normal individuals. Sequestration of activated CD4+ memory T cells in the atopic skin may be responsible for the increased ratio.

As hypothesized, we were able to identify increased lymphocyte responsiveness by atopic MD dogs to crude M pachydermatis extract, compared with clinically normal dogs. We were surprised to also find a significant decrease in the PBMC response of the atopic MO dogs, compared with the atopic MD dogs, despite the extremely high numbers of M pachydermatis typically found in the external ear canal exudates of dogs with otitis externa. Potential explanations for this finding include differences in the skin surface microclimate between the external ear canal and haired skin and differences in the T-cell populations of these respective areas. To our knowledge, the latter has yet to be investigated. Regarding microclimate, the surface of the inflamed external ear canal epithelium of atopic dogs is rich in wax and lipids. This environment favors proliferation of lipophilic organisms like M pachydermatis but could also conceivably contribute to the epithelial surface barrier function by discouraging microbial adherence and penetration. We speculate that such function could therefore inhibit recognition of the organism by the local T-cell population.

Significant differences were not found in PBMC response amongst the other groups of dogs (ie, atopic MD vs atopic control dogs; atopic control vs clinically normal dogs). Several potential reasons for our failure to detect differences among these groups exist. The most plausible explanation for reactivity in the atopic control and clinically normal dogs is prior episodes of MD and exposure to normal commensal numbers of the organism (that do not meet our cytologic criteria for a positive yeast count), respectively.

In clinically normal animals, the T-cell population is responsible for the continual surveillance of the skin’s surface environment, and a clonal population of T cells should exist that is specific for commensal organisms like M pachydermatis and Staphylococcus bacteria. At the same time, normal skin barrier functions such as temperature, humidity, pH, soluble surface proteins (immunoglobulins, transferrin, and complement components), lipids, and normal structural features of the epidermis and hair follicles serve to exclude microbial penetration during homeostasis.

Disruption of normal skin surface barrier function by the self-trauma associated with pruritus may allow exposure of a primed atopic immune response to microbial antigens. In the paradigm for atopy, expansion of these clonal populations, even in the absence of microbial overgrowth, should be suspected.

Although the inflammatory reaction to M pachydermatis in dogs with AD is intense, a role for superantigens in MD in dogs has not yet been investigated. Superantigens are microbial products capable of binding a large repertoire of T-cell receptors that share common Vβ regions without regard for other binding determinants. This allows activation of as much as 5 to 30% of the entire T-cell population. It has recently been shown that M furfur does not play a superantigenic role in humans with AD.

It is also plausible that the crude extract of M pachydermatis that we used did not contain a high enough concentration of a major allergen (ie, a dilutional effect) to result in differential PBMC reactivity between atopic MD and atopic control dogs. A major allergen is an antigenic epitope recognized by ≥ 50% of an allergenic population. Currently, 9 allergens of M furfur recognized by IgE from the sera of atopic humans have been characterized. Finally, because our crude extract was filtered prior to use in the lymphocyte blastogenesis assay, we cannot rule out the possibility that some antigenic determinants were incompletely soluble (particulate) proteins or glycoproteins that were excluded by the filtration process.

In humans with seborrheic dermatitis, a disease characterized by increased surface scale and lipid content but that is not associated with an atopic phenotype, a difference is not found in T-cell reactivity to M furfur, compared with clinically normal humans. A similar condition (termed idiopathic seborrheic dermatitis) is found in several dog breeds (including Basset Hounds) and also promotes M pachydermatis overgrowth. Seborrheic Basset Hounds with high M pachydermatis counts on the skin had significantly lower PBMC reactivity to M pachydermatis extract than did clinically normal Basset Hounds, suggesting an impaired cell-mediated immune response to the yeast in this condition. However, the seborrheic Basset Hounds with MD did not differ from clinically normal Beagles or Irish Setters. As one plausible explanation, the authors of this study suggest a potential suppressive role of Staphylococcus intermedius, which has been shown to impair T-cell reactivity to Demodex canis antigens during coinfection. Certainly, many of our atopic dogs had concurrent surface bacterial overgrowth, or superficial pyoderma (data not shown), but had the opposite T-cell response. These findings, considered in parallel, support a role for T-cell hyperresponsiveness to M pachydermatis as part of the paradigm for AD in dogs.

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