

Allergenic cross-reactivities in flea-reactive canine serum samples

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Objectives—To determine whether flea extract could be determined (via ELISA) to share allergenic epitopes with other insects, and to determine whether sera with different reactivities to insect extracts have different cross-reactivity patterns.

Sample Population—69 canine serum samples that were selected from samples submitted for routine ELISA allergy testing and had previously been found to have high reactivities to flea.

Procedure—Each serum sample was assessed by means of a direct ELISA for IgE binding to 11 common insects. Samples that were reactive primarily to flea extract alone were designated pool 1, samples that were reactive to small numbers of insects were designated pool 2, and samples that were reactive to all or almost all insects were designated pool 3. Samples that did not have any apparent patterns of cross-reactivity were not included in the rest of the study. Inhibition ELISA techniques were used with the 3 serum pools to determine whether multiple insect extracts inhibited reactivity on flea-coated ELISA plates. Those extracts were used to coat ELISA plates, and reciprocal inhibition studies were then performed.

Results—Black fly, black ant, and cockroach extracts were capable of > 50% inhibition of flea solid-phase IgE binding with all 3 serum pools. In the ELISA inhibition studies, flea extract was able to inhibit IgE binding to each extract with all pools, confirming reciprocal inhibition.

Conclusions—Inhibition of IgE binding to solid-phase flea antigen by black ant, black fly, and cockroach extracts suggested sharing of allergenic epitopes among these species. Reciprocal inhibition studies further confirmed these findings. These results indicated in vitro cross-reactivity between flea, black ant, black fly, and cockroach extracts. These results need to be further investigated in vivo.

Clinical Relevance—It is possible that dogs may become sensitized to fleas via exposure to other insects, and that flea allergenic dogs may have signs of pruritus, in the absence of fleas, if exposed to cross-reactive insects. (*Am J Vet Res* 1996;57:1000–1005)

Flea allergy dermatitis is the most common allergic skin disorder in dogs.^{1,2} It plays a major role in at least 50% of all dermatologic conditions, and often co-

exists with other allergic conditions,² complicating diagnosis. In recent years, allergic dermatitis related to insects other than fleas has been described in dogs.³ It is thought that dogs may become sensitized and exposed to insects or insect antigens through various routes.²

Use of ELISA to evaluate serum samples from dogs for IgE to biting flies (*Culicoides* spp) has revealed that many dogs have high concentrations of IgE not only to biting flies, but also to multiple related and unrelated biting and nonbiting insects.⁴ Further evaluation of these samples via inhibition techniques revealed that some insect extracts were able to competitively inhibit binding of IgE to immobilized *Culicoides* antigens. This finding suggests that these insects may contain similar antigenic structures that cause cross-reactivity in vitro.⁴ In vivo cross-reactivity to multiple insects has not yet been demonstrated in dogs, but is a well known phenomenon in human beings,^{4,5} and may be evident during skin testing, or may be associated with production of clinical signs of hypersensitivity upon challenge exposure to an allergen to which a person is naive, but which shares important allergenic determinants with a substance to which that person has already been sensitized.

Preliminary studies in dogs have been undertaken by 2 of the authors (TJG, REE) to evaluate the relationship between flea IgE specificity and specificity of IgE for other insects. Results of ELISA inhibition studies suggested that such cross-reactivities between fleas and other insects may be prevalent.

The purpose of the study reported here was to test canine serum samples that had high concentrations of flea-specific IgE for reactivities to antigens from various biting and nonbiting insects. We further attempted to characterize the antigenic similarity between fleas and some of these other insects by determining, by use of ELISA, whether certain insect extracts would inhibit reactivity to fleas and whether a flea extract would inhibit reactivity to these other insects.

Materials and Methods

Sera—Sixty-nine canine serum samples were selected from samples submitted for routine immunologic testing for IgE concentrations to various allergens. All serum samples had been protected from bacterial contamination by addition of sodium azide (final concentration, 0.02%) upon arrival at the testing laboratory, and were stored at 2 to 8 C until tested. All samples were found, by use of a direct-binding ELISA, to have IgE reactivity to flea antigen at least half that of a reference sample. This reference sample is a highly diverse pool of canine serum samples from dogs with well documented reactivity to fleas or multiple allergens, as determined by means of skin testing, or with a history and clinical signs of atopy or allergic skin disease, as determined by a veterinarian. This ref-

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erence sample has been shown to contain IgE antibodies to > 100 allergens, including all major trees, grasses, weeds, molds, and insects, by ELISA or immunoblot analyses.

Determination of IgE reactivity to insects—Serum samples were tested, by means of a direct ELISA, for IgE reactivity to fleas and 10 additional biting and nonbiting insects. Individual columns of polystyrene microtitration plates^b were coated overnight at 2 to 8 C with 300 μ l of saturating concentrations (1 to 10 μ g of protein/mL in 0.04M carbonate/bicarbonate buffer, pH 9.6) of whole body extracts of each of the following insects: house fly (*Musca domestica*), horsefly (*Tabanus* spp), deerfly (*Chrysops* spp), caddisfly (*Trichoptera* spp), black fly (*Simulium* spp), black ant (*Camponotus* spp), fire ant (*Solenopsis* spp), moth (*Heterocera* spp), mosquito (*Aedes* spp), cockroach (*Periplaneta americana* and *Blattella germanicus*), and flea (*Ctenocephalides* spp). The saturating concentrations for each allergen had been previously determined by means of dose-response studies, using the flea-positive reference serum at a dilution of 1:50. An antigen-free control column containing only carbonate buffer was included on each plate. Coated plates were washed with phosphate-buffered saline solution (PBSS; 10 mM sodium phosphate, 1.5 mM potassium phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, 0.02% sodium azide, pH 7.2), then incubated with 200 μ l of each serum sample diluted 1:10 in PBSS containing 0.05% Tween 20 and 0.29M sodium chloride. Flea-positive reference serum and negative (5 mg/ml dog albumin) control samples that had been diluted similarly were included on each plate. Plates were incubated for 4 to 6 hours at room temperature (22 to 24 C), washed with PBSS, then incubated overnight at room temperature with 200 μ l of biotinylated rabbit anti-canine IgE diluted 1:4,000 in PBSS containing 0.05% Tween 20 and 0.5% calf skin gelatin.^c In direct binding studies previously performed by 2 of the authors (TJG, REE), the biotinylated rabbit anti-canine IgE was found to not recognize canine IgG or canine IgM, which indicated that it was specific for IgE. The following morning, plates were washed with PBSS and incubated at room temperature for 1 hour with 200 μ l of alkaline phosphatase-labeled avidin^d diluted 1:200 in PBSS containing 0.05% Tween 20 and 0.5% calf skin gelatin. Plates were washed with PBSS and incubated a final time with 200 μ l of 1 mg/mL para-nitrophenyl phosphate in 1.0M diethanolamine (pH 10.2) for 30 to 60 minutes. Absorbance was read at a wavelength of 405 nm after 5 and 30 minutes of substrate incubation. Values were subtracted to yield absorbance differences for each well. Samples for which absorbance was > 50% of the positive control sample were considered to have reactivity to that insect extract. The ELISA procedure was closely related to that described for analysis of human⁶ and canine serum samples.⁷

For the ELISA inhibition study, microtitration plates were coated with a saturating concentration of each insect extract in carbonate buffer as described for the direct ELISA. Separate evaluations were performed for each serum pool. Plates were then washed with PBSS, and 200 μ l aliquots of the serum pools diluted 1:10 to 1:50 with PBSS in 0.05% Tween 20 and 0.29M NaCl containing various concentrations (0.05 to 100 μ g protein/mL) of insect extract, including the same insect extract used to prepare the solid phase for that plate, were added. Plates were incubated for 4 to 6 hours at room temperature. Subsequent steps in the assay procedure (washing and incubation with biotinylated anti-canine IgE, alkaline phosphatase-avidin, and para-nitrophenyl phosphate) were performed as described for the direct ELISA, and absorbance was read 15 and 30 minutes after addition of substrate. Samples were run in duplicate and mean values were calculated.

Inhibition percentage was calculated by use of the formula: percentage inhibition = $100[(\text{absorbance of uninhibited serum}) - (\text{absorbance of inhibited serum})]/(\text{absorbance of uninhibited serum})$. The slope of the inhibition curve was calculated by use of the formula: slope = change in per-

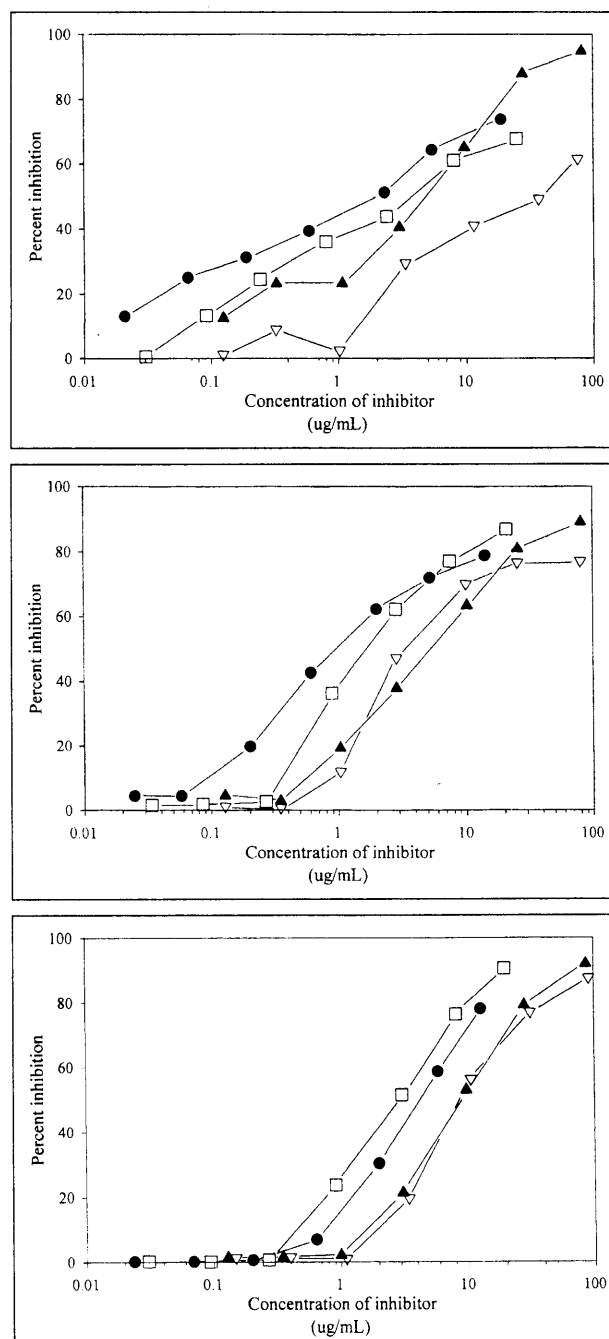


Figure 1—Inhibition curves produced by adding flea (▲), black fly (●), black ant (□), or cockroach (▽) extracts to assays designed to detect binding between solid phase flea extract and IgE in canine serum samples primarily reactive to flea extract (top), serum samples primarily reactive to fleas and small numbers of other insects (middle), and serum samples reactive to fleas and large numbers of other insects (bottom).

centage inhibition/change in concentration of inhibitor. Reciprocal inhibition studies of insect reactivity with flea extract were then performed. For this study, the 3 extracts that were found to be the best inhibitors of IgE binding to flea extract (black fly, black ant, and cockroach) were used as the solid phase, and reactivity was inhibited with flea extract.

Results

Storage of the serum at 2 to 8 C for various periods of time prior to testing resulted in some decrease

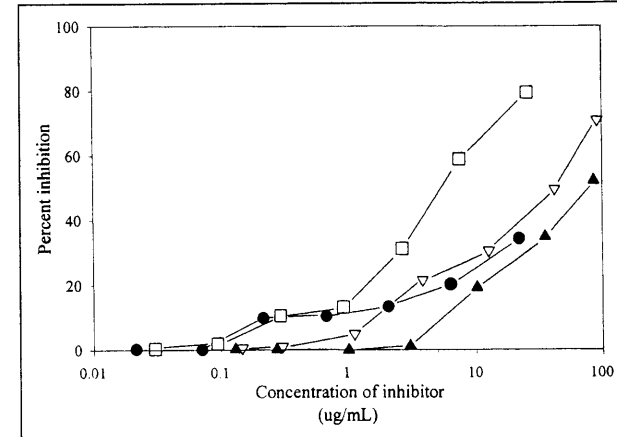
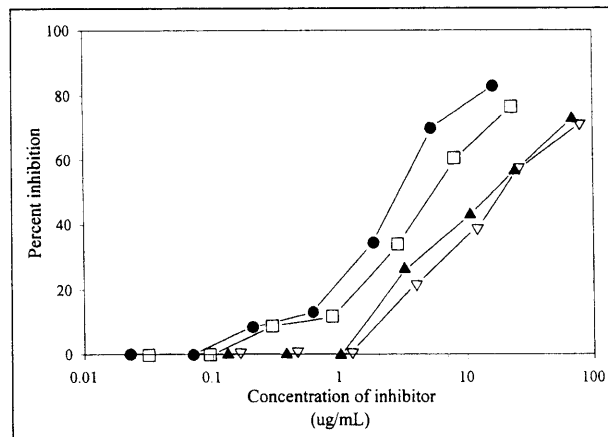
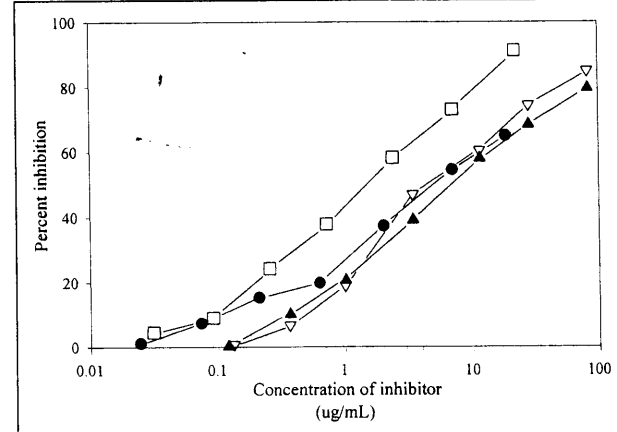
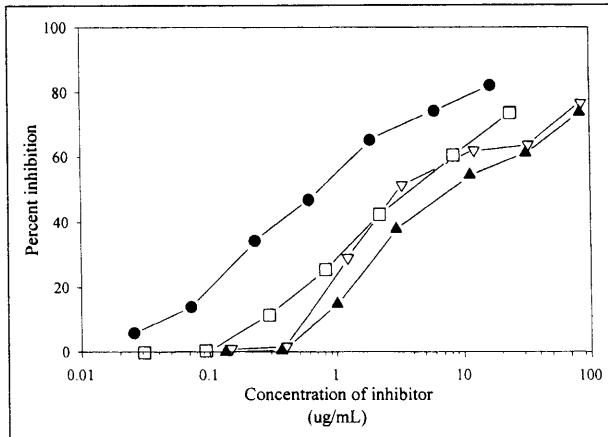
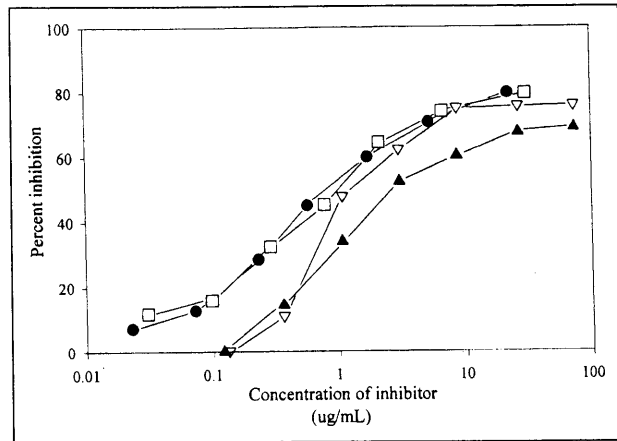
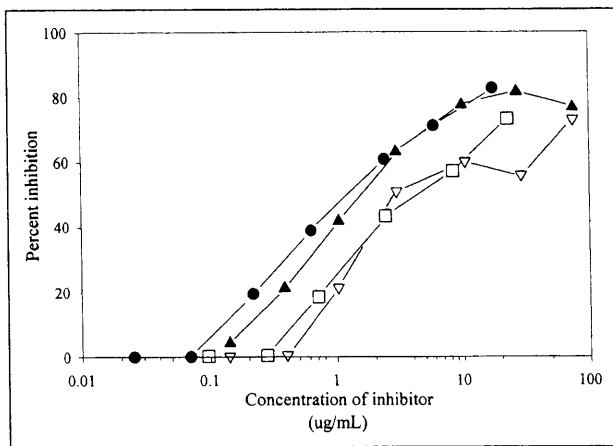


Figure 2—Inhibition curves produced by adding flea (▲), black fly (●), black ant (□), or cockroach (▽) extracts to assays designed to detect binding between solid phase black fly extract and IgE in canine serum samples primarily reactive to flea extract (top), serum samples primarily reactive to fleas and small numbers of other insects (middle), and serum samples reactive to fleas and large numbers of other insects (bottom).

Figure 3—Inhibition curves produced by adding flea (▲), black fly (●), black ant (□), or cockroach (▽) extracts to assays designed to detect binding between solid phase black ant extract and IgE in canine serum samples primarily reactive to flea extract (top), serum samples primarily reactive to fleas and small numbers of other insects (middle), and serum samples reactive to fleas and large numbers of other insects (bottom).

in IgE reactivity toward flea antigen, compared with reactivity determined when the samples were first obtained. However, all samples retained most of their original reactivity, and none of the samples retained < 30% of the flea IgE reactivity observed for the positive-control reference pool sample. Any detrimental effect that storage had on the reactivity of samples that had been stored for longer periods was minimized by pooling with samples that had been stored for shorter periods. In addition, decreases in IgE reactivity to insects

other than flea were assumed to be similar to the decreases in reactivity to flea.

Evaluation of results of the direct ELISA revealed wide variations in reactivities to the 11 insect allergen extracts. Four patterns of reactivity were identified. Three samples had IgE reactivity to flea extract, but did not react as well with any of the 10 other insect extracts. These samples were combined and designated as pool 1. Eighteen samples had reactivity to distinct groups (eg, flies or ants) of small numbers of insect extracts, as well

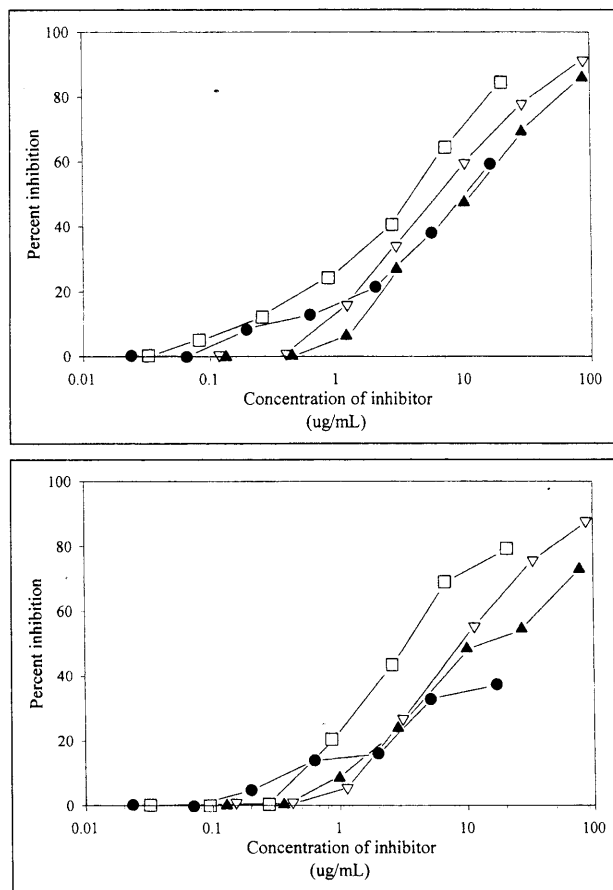


Figure 4—Inhibition curves produced by adding flea (▲), black fly (●), black ant (□), or cockroach (▽) extracts to assays designed to detect binding between solid phase cockroach extract and IgE in canine serum samples primarily reactive to fleas and small numbers of other insects (top) and serum samples reactive to fleas and large numbers of other insects (bottom).

as to flea extract. These samples were combined and designated as pool 2. Nineteen samples had reactivity to at least 6 insect extracts. These 19 samples were screened, by means of an ELISA, for reactivity to at least 22 inhalant allergens, including multiple grasses, trees, weeds, and molds. Two of the 19 were found to react nonspecifically to nearly all of the inhalant allergens, and were eliminated from further study. The remaining 17 samples were combined and designated as pool 3. The fourth reactivity group (29 samples) had patterns of random and low-magnitude IgE specificity. These sera were not investigated further in this study. However, although reactivity profiles for sera such as these appear to be random and not in defined groups, further investigations may reveal important IgE binding patterns.

For all 3 serum pools, extracts of black ant, black fly, and cockroach, when applied at concentrations $> 10 \mu\text{g/ml}$, consistently displayed $> 50\%$ inhibition of IgE binding to a flea solid phase (Fig 1). Black ant and black fly caused 50% inhibition at lower concentrations than did flea or cockroach. Slopes of the inhibition curves for flea, black ant, black fly, and cockroach were not similar to each other in all cases, supporting the notion that although shared or common antigens may exist among these insects, allergenic components unique to these species may be present as well.

Reciprocal inhibition of insect reactivity was demonstrated by adding black fly, black ant, cockroach (the 3 best inhibitors of IgE binding to flea extract), and flea extract to microtitration plates coated with black fly (Fig 2), black ant (Fig 3), or cockroach (Fig 4) extract. All combinations were examined except for cockroach-coated plates, using pool 1, owing to an insufficient volume of this serum pool. In all cases examined, flea extract at concentrations $> 100 \mu\text{g/ml}$ resulted in $> 50\%$ inhibition of IgE binding to the solid phase insect extracts. Black ant caused $> 50\%$ inhibition of IgE binding to cockroach and black ant solid phases at lower concentrations than did the other extracts. Black fly caused more inhibition on the black fly-coated plates than did the other extracts.

Discussion

Measurement of the degree to which allergenic extracts inhibit binding of IgE to solid phase antigens has been a useful tool in human allergy research for more than 20 years.⁸ Inhibition of IgE binding implies that the 2 compounds have some degree of similarity in antigenic structure.^{4,8,9}

One method of estimating the potency of inhibition by an allergenic extract is to calculate the concentration of extract needed to produce 50% inhibition of IgE binding.⁹ The lower the protein concentration required to produce 50% inhibition, the more potent the inhibitor. However, this may not be an ideal method of assessing inhibition potency, because substances other than proteins may play a role in inhibition. For example, an extract that demonstrates $\geq 50\%$ inhibition at low protein concentrations may actually contain high concentrations of allergenic nonprotein components. In addition, potency of inhibition depends not only on the concentration of the allergenic substances,⁹ but also on the affinity of IgE for the inhibitor versus the solid phase antigen.¹⁰ A more potent inhibitor may have higher binding affinity for IgE, or, as is more often the case, may simply have more critical allergenic protein per milligram of total protein or dry weight. In some cases, an inhibitor that shares antigenic determinants with the solid phase antigen may have a greater affinity or more relevant binding sites for IgE than does the solid phase allergen.¹⁰

Another method of examining potency of inhibition is to examine the slope of the percentage inhibition/inhibitor concentration curve. The mid-point of the curve is usually of greatest interest, because most curves are sigmoidal. The slope between 30 and 70% inhibition is the most relevant, and the slope at 50% inhibition is usually used for comparative purposes.^{8,9} Production of similar inhibition curves by 2 substances indicates that there is a high degree of antigenic similarity between the substances.^{4,8,9} However, a substance that is a potent inhibitor of IgE binding, indicating productive binding interactions across multiple allergenic structures, may not necessarily be similar to the solid phase antigen when examined in toto.

Reciprocal inhibition studies are used to further elucidate the nature of the antigenic similarity between 2 extracts. Ideally, the inhibition and reciprocal inhibition curves should be similar for a given extract (ie, flea should inhibit IgE binding to black ant in a similar fashion to the manner in which black ant inhibits IgE binding to flea). The curves may differ, however, be-

cause of differences between the antigenic structures for extracts in the liquid phase and the structures for extracts in the solid phase. Molecular conformation may change when proteins bind to plastic. The proteins may "flatten out," exposing previously hidden binding sites, or may preferentially bind to plastic in a fashion that may prevent antibody access to some antigenic determinants. Thus, differences between the inhibition and reciprocal inhibition curves can give information on the relationship between the solid and liquid phases of the extracts, as well as provide a better idea of the true antigenic similarity between extracts.

In people, in vitro cross inhibition of IgE binding between allergens often correlates with cross-reactivity in vivo.^{4,5} Patients may demonstrate skin test reactions, or even clinical signs, when exposed to agents to which they have not previously been exposed, if those agents are similar enough to other substances to which the patients are hypersensitive.^{4,5}

A major objective of this study was to ascertain patterns of reactivity towards different insect extracts for flea reactivity-positive serum samples. We detected several patterns, but were surprised to find that only 3 of 69 serum samples reacted only with flea extract. The remaining serum samples all reacted to at least 1 other insect extract. Many of these reacted to a few insects, but we selected those that reacted to small groups of insects (eg, flies or ants) and flea. We were also surprised to find that 19 samples reacted to almost all insect extracts, as true panreactivity would be expected to be uncommon. This finding tends to confirm our original suspicion that there is cross-reactivity between relevant allergens of insects, and suggests that the major relevant proteins of flea may be the same as or similar to proteins of other insects.

It is possible that the serum samples that reacted only to or predominantly to the flea extract contained IgE primarily targeted toward minor proteins that are not shared with other insects. A similar phenomenon has been reported in human beings. For instance, although most human patients who are hypersensitive to a fungus tend to be sensitive to all fungi known to be antigenically related, a small number will react only to a small number of fungi.⁴

We cannot ignore the possibility, however, that the panreactivity detected for some samples could be, in part, a result of cross-reactivity with noninsect allergens. For example, if certain insects cross-reacted with a common allergen, such as housedust mite, one might expect any housedust-sensitive individual to have multiple insect reactivities in vitro. Samples used in this study were not controlled specifically for noninsect reactivities, and, on initial assays, most did have reactivities to multiple environmental allergens. However, cross-reactivity with noninsect allergens should not affect the results of the inhibition assays. In addition, in studies performed on canine serum samples with insect reactivities similar to those for samples used in this study, a variety of pollens were not able to inhibit immunoglobulin binding to various insect extracts (Grier TJ, Esch RE: Unpublished data, 1994). Further studies using serum samples from dogs with signs of only flea hypersensitivity could potentially be useful to further evaluate the possibility of interference by noninsect allergens.

A second objective of this study was to determine whether certain insect extracts could inhibit flea reactivity. We found that 3 extracts (black ant, black fly, and cockroach) were able to accomplish this consistently for all pools tested. Again, this would imply at least some antigenic similarity between their component structures.

A final objective of this study was to determine whether those extracts that were able to inhibit IgE binding to flea extract would in turn be inhibited by flea extract. We found that flea extract was able to inhibit IgE binding to each of these extracts by > 50% consistently. This result helps to confirm the assumption of antigenic similarity between flea and black ant, black fly, and cockroach, although the antigenic determinants are not identical in toto, as evidenced by the dissimilarity between the slopes of the inhibition-concentration curves.

There are several major implications to these results. Inhibition by an extract often (but not always) implies in vitro cross-reactivity. This may explain why some canine serum samples react to extracts of antigens to which the dogs may never have been exposed. Our results suggest that such reactions should be understood to represent unavoidable limitations of the assay, rather than evidence that the assay is flawed. Without inhibition studies, it cannot be determined whether multiple reactions in individual serum samples are a result of independent sensitization or a result of cross-reactivity. It should be stressed that skin test reactions are also prone to this problem. Although it is true that nonimmunologic mechanisms can potentially produce inhibition of immunoglobulin binding to a solid phase antigen, the use of reciprocal inhibition studies helps to minimize the possibility of such an occurrence going unnoticed, as does the proper use of known positive and negative controls in inhibition studies.

This distinction may be of little importance, however, if what is known to be true in human beings is also true in dogs. It is known that people with in vitro cross-reactivity occasionally have in vivo cross-reactivity; that is, they may develop allergic symptoms when exposed to cross-reacting allergens to which they have never previously been exposed.⁵ Clinical trials to prove if this effect can be demonstrated in dogs are needed.

One possible clinical implication of this study is that, in dogs with IgE cross-reactivity in vitro, insects other than fleas may have the potential to cause pruritus. Thus, a flea-allergic dog may still have clinically significant pruritus when fleas are eliminated, if persistently exposed to other insects.

It is interesting to consider that dogs might actually be sensitized to fleas by exposure to black ants, or other cross-reacting insects. Although the phenomenon of more-rapid sensitization to fleas in flea-naïve or intermittently exposed animals is well documented,¹¹ our results offer a possible explanation for why this phenomenon might occur.

*Grier TJ, Willis EL, Esch RE, et al. Canine insect hypersensitivity: immunochemical evidence for common or cross reactive antigens, in *Proceedings*. 10th Annu Meet Am Acad Vet Dermatol and Am Coll Vet Dermatol 1994;21.

^bImmulon II, Dynatech Laboratories, Chantilly, Va.

^cICN, Aurora, Ohio.

^dZymed, South San Francisco, Calif.

^eAmresco, Solon, Ohio.

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