

THE BINDING OF MOUSE HYBRIDOMA AND HUMAN IgE ANTIBODIES TO THE MAJOR FECAL ALLERGEN, *Der p* I, OF *Dermatophagoides pteronyssinus*

Relative Binding Site Location and Species Specificity Studied by Solid-Phase Inhibition Assays with Radiolabeled Antigen

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The relative binding site location and species specificity of 19 mouse hybridoma antibodies, produced in four laboratories, to *Dermatophagoides pteronyssinus* major fecal allergen, *Der p* I, was studied by using immobilized mAb and inhibitions of radiolabeled Ag binding. Four mAb groups were defined, within which 4, 6, 8, and 5 mAb, respectively, cross-inhibited each other. Five mAb were members of both group 2 and 3, demonstrating a considerable overlap of epitopes between the corresponding antibody-binding regions. The degree of mAb species specificity, as assessed by inhibition with cold *Der p* I and Ag *Der m* I and *Der f* I from the related species, *Dermatophagoides microceras* and *Dermatophagoides farinae*, was highly variable even for mAb binding to the same region on the Ag. Five cases of cross-reactivity between *Der p* I and *Der m* I and one case of cross-reactivity between *Der p* I and *Der f* I were found. The N-terminal 30 amino acids of the three species showed 7 substitutions between *Der p* I and *Der m* I/*Der f* I and 2 between *Der f* I and *Der p* I/*Der m* I. Single mAb inhibited up to 65% of labeled *Der p* I binding to immobilized human IgE from allergic patients' sera and up to 24% of labeled *Der p* I binding to immobilized rabbit antibodies. The spectrum of species specificities in human IgE sera, as assessed by inhibitions with cold Ag, was similar to that of the mAb. No evidence for the presence of strictly sequential epitopes, reactive with either mAb or human IgE was found, as judged from the weak inhibitory activity of acid-denatured *Der p* I.

House dust mites of the genus *Dermatophagoides* are cosmopolitan inhabitants of human dwellings (1), causing IgE-mediated hypersensitivity (asthma and/or rhinoconjunctivitis) in susceptible individuals (2, 3). Three related species, *Dermatophagoides pteronyssinus*, *Dermatophagoides microceras*, and *Dermatophagoides farinae* occur with variable frequencies (1, 4). Their ma-

for fecal allergens, *Der p* I, *Der m* I, and *Der f* I have been purified and characterized physicochemically in several laboratories (5-10). The allergenic molecules are readily immunogenic in rabbits and mice (7, 9, 11, 12), and humans subjected to natural or artificial immunization (hyposensitization) exhibit variable titers of specific IgE, IgG, and IgA in their serum (13-17). Changes in levels of allergen-specific IgE or IgG have hitherto been poor indicators for the highly variable, protective effect of immunotherapy in this (15) as well as in other inhalation allergies (18). A more detailed knowledge of the repertoire of epitopes for antibodies and TCR (19) is probably necessary before conditions for specific modulation of the immune response can be settled.

In the present study, the relative binding site location and species specificity of 19 mouse mAb were established and their binding pattern compared with that of IgE antibodies from 10 naturally exposed allergic humans. The experiments involved the same labeled reagent (radiolabeled *Der p* I) and similar assay designs for the mouse mAb and the polyclonal human IgE.

MATERIALS AND METHODS

mAb. Eighteen mAb against *D. pteronyssinus* major allergen, *Der p* I, and two mAb against *Der f* I from *D. farinae* were obtained from four laboratories (Table I). Six mAb (Nhp1 to Nhp6) were produced in our laboratory (12). In short, spleen cells of BALB/c mice, immunized with partially purified *Der p* I (AML³ preparation) were fused with X63-Ag8.653 myeloma cells and clones were screened by an enzyme immunoassay by employing immobilized rabbit anti-mouse IgG and horse radish peroxidase-labeled *Der p* I. Purified Ig for coating PVC trays were produced from ascites by IEF (Nhp1 to 5) or by absorption of pooled supernatants (Nhp6) to mercaptoethanol/vinylsulfone-agarose (T-gel; Kem-En-Tec, Copenhagen) in 1.5 M ammonium sulfate and elution with 0.1 M NaCl.

Two mAb against *Der p* I (10B9F6 and 5H8C12) (11) and two against *Der f* I (4C1B8 and 6A8B10) (8) were gifts from Dr. M. Chapman, Division of Allergy and Clinical Immunology, University of Virginia. They were received as freeze-dried, dialyzed, 50% sodium ammonium sulfate precipitates of ascites and were reconstituted as 10 mg/ml.

Nine mAb against *Der p* I (A2.2/79, B2.1/35, B2.1/81, C3.6/104, C3.6/62, C4.1/96, C4.1/40, C4.1/102, and HY3/90) were gifts from Dr. G. Delespesse, Hôpital Notre Dame, Montreal. They were received as twice sodium ammonium sulfate-precipitated ascites, dialyzed and freeze-dried and were reconstituted at 10 mg/ml.

One mAb⁴ (Alb2) against *Der p* I obtained as ascites, was a gift from Dr. R. Aalberse, The Central Laboratory of the Netherlands

³ Abbreviations used in this paper: AML, partially purified allergens *Der p* I/*Der m* I/*Der f* I; PVC, polyvinylchloride; C_{50%in}, concentration of inhibiting antibody or Ag eliciting 50% inhibition of labeled Ag binding; PTH, phenylthiohydantoin.

⁴ van der Zee, J. S., P. van Swieten, H. M. Jansen, and R. C. Aalberse. Skin tests and histamine release with P1-depleted *D. pteronyssinus* body extracts and purified P1. Submitted for publication.

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TABLE I
Inhibition of labeled *Der p 1* binding to immobilized mAb by liquid-phase inhibiting mAb^a

Inhibitors	Immobilized mAb												
	4C1B8	Nhp1	10B9F6	Nhp4	Nhp3	A2.2/79	5H8C12	Alb2	Nhp2	Nhp6	Nhp5	B2.1/81	C4.1/40
4C1B8	3+	3+	2+	.	.	.	.	.	.	.	.	.	.
Nhp1	.	3+	2+	.	.	.	.	.	.	+	.	.	.
10B9F6	+	4+	4+	2+	.	.	.	.	.	.	.	.	.
Nhp4	2+	4+	4+	3+	2+	.	.	.	.	2+	.	.	.
Nhp3	.	.	.	.	4+	4+	4+	.	.	.	.	.	.
A2.2/79	.	.	.	.	2+	3+	3+	3+	4+	2+	.	.	.
5H8C12	.	.	.	.	+	2+	2+	2+	+	+	.	.	.
Alb2	.	.	.	.	.	2+	3+	2+	3+	2+	.	.	.
Nhp2	.	.	.	.	.	+	2+	2+	2+	+	.	.	.
Nhp6	+	+	.	.	.	2+	3+	2+	2+	2+	.	.	.
Nhp5	.	.	.	.	.	.	.	.	.	+	4+	3+	3+
B2.1/81	.	.	.	.	.	.	.	.	.	.	+	4+	2+
C4.1/40	.	.	.	.	.	.	.	.	.	.	2+	3+	4+
6A8B10	.	.	.	.	.	.	.	.	.	.	.	.	.

^a mAb (0.8 to 7.8 $\mu\text{g/ml}$, except Nhp3: 0.3 $\mu\text{g/ml}$) were coated onto PVC trays. Binding of labeled *Der p 1* (5 ng) was inhibited with dilutions of raw (Nhp1 to 6, Alb2) or purified ascites. 4+, 50% inhibition of binding at $<0.01 \mu\text{g Ig/ml}$; 3+, 50% inhibition of binding at $<0.1 \mu\text{g Ig/ml}$; 2+, 50% inhibition of binding at $<1 \mu\text{g Ig/ml}$; +, 50% inhibition of binding at $<10 \mu\text{g Ig/ml}$; ., $<50\%$ inhibition of binding at $10 \mu\text{g Ig/ml}$.

Red Cross Blood Transfusion Service, Amsterdam.

Two mAb were used for control of nonspecific binding or inhibition. Fd-1, directed against a major cat allergen, and Alb4, directed against a different antigen of *D. pteronyssinus*, were obtained from Dr. R. Aalberse.

All mAb received except 5H8C12 and B2.1/35 were found to be IgG1. Ig concentrations in reconstituted mAb and ascites were determined by single radial immunodiffusion by using rabbit anti-mouse IgG1 antibodies (Nordic Immunological Labs, Tilburg, The Netherlands). A reference mouse serum with known concentration of IgG1 was a gift from M. Kærving-Broe, Dakopatts, Glostrup, Denmark.

Rabbit antibodies. Two rabbits (R1 and R2) were hyperimmunized with biweekly injections s.c. of purified *Der p 1* (25 μg) in IFA. Affinity purification of specific antibodies took place as previously described (20).

Human IgE sera. Seven sera (Br) from adult mite-allergic patients originated from a previous study (14) in Brussels, Belgium. The sera were selected from a larger population and were those with the most prominent reactions to *D. pteronyssinus* in radioallergosorbent test (21) and to *Der p 1* in crossed radioimmunoelectrophoresis (22). Three sera (UW) from mite-allergic children with asthma originated from a previous study in Bochum, West-Germany.⁵ The sera used were taken before the initiation of immunotherapy. The predominant sensitization in both study areas was against *D. pteronyssinus*, as measured by a species-specific ELISA (20) for dust content of mite Ag in the patients' habitations. Normal serum was a pool from 1000 Danish blood donors.

Ag. *Der p 1*, *Der m 1*, and *Der f 1* were produced by acetone extraction and precipitation of excrement fractions from mite cultures followed by hydrophobic interaction chromatography as previously described (6, 7). The names of major allergens (previously Dp42, Dm6, and Df6) has been changed to follow the recommendations of the International Union of Immunological Societies (23). Partially purified *Der p 1*, *Der m 1*, and *Der f 1* (AML fractions) for use as inhibitors in Ag-binding assays were produced as previously described (7, 24). Concentrations of *Der p 1*, *Der m 1*, and *Der f 1* were determined by rocket immunoelectrophoresis (22) by using monospecific rabbit antibodies. Acid-denatured *Der p 1* was produced by dissolving 2 mg Dp-AML in 1 ml 1.0 M sodium citrate buffer, pH 2.0 with neutralization after 3 h with 0.4 M sodium hydroxide. The denatured preparation produced one dominant band in SDS-PAGE at the same position as did native AML and pure *Der p 1* (M, approximately 28 kDa), but gave no reaction with rabbit anti-*Der p 1* in rocket immunoelectrophoresis.

Radiolabeled Ag. Iodination of *Der p 1*, *Der m 1*, and *Der f 1* with ¹²⁵I took place by the chloramine-T method (25). Unbound iodine was separated from protein by Sephadex G-25 gel filtration. Ag recovery and spec. act. was assessed by rocket immunoelectrophoresis. Labeled precipitate was cut from the wet agarose gel and counted. After subtraction of the counts obtained from an equally sized, anodically positioned gel section, the specific activity of immunochemically

reactive *Der p 1* was calculated as 9.2 $\mu\text{Ci}/\mu\text{g}$.

Binding of labeled Ag to immobilized antibodies. mAb or rabbit antibody (100 μl) diluted in 0.1 M sodium carbonate buffer (pH 9.8) was immobilized overnight at ambient temperature on PVC micro-titer trays (Titertek Immunoassay Plate, Flow Laboratories). After three 3-min washings in PBS with 0.05% Tween-20, 100 μl radiolabeled *Der p 1* in PBS with 0.5% BSA were added for incubation overnight at ambient temperature. After 5 washings (the first two with separate aspiration of single wells) in PBS/Tween-20, the wells were cut out with scissors and placed in premarked tubes for gamma counting.

For binding of labeled *Der p 1* to immobilized human IgE, PVC trays were coated with 100 μl rabbit anti-human IgE, specific for the ϵ -chain (26), dissolved at 5 $\mu\text{g/ml}$ in sodium carbonate buffer (pH 9.8) overnight at ambient temperature. After three 3-min washings in PBS/Tween-20, patient serum and normal serum was added for incubation overnight. After three 3-min washings in PBS/Tween-20, 5 ng radiolabeled *Der p 1* were added for a 6-h incubation period. Dilutions of patient sera (1/2 to 1/10) for inhibition assays were chosen so as to give bound activity 10 times that of normal serum.

Inhibition assays. Mutual inhibitions of mAb were performed by mixing 5 ng labeled Ag in 50 μl with 50 μl dilutions (10^{-2} to 10^{-7}) of the inhibiting mAb in PBS/BSA immediately before incubation with the immobilized mAb. Dilutions of raw ascites (Nhp1 to Nhp6, Alb2, Alb4, and Fd1) or reconstituted, purified ascites (10 mg/ml) were used. IgG concentrations as determined by single radial immunodiffusion were employed for comparisons of inhibitory potency. For two purified mAb (5H8C12 and B2.1/35), a 50% IgG/dry weight was estimated being the average value for the other 11 determinations.

mAb inhibition of labeled Ag binding to immobilized IgE (individual or pooled sera) and to rabbit anti-*Der p 1* was performed by mixing 5 ng labeled *Der p 1* in 50 μl with 50 μl of eight selected mAb (Nhp2 to Nhp5, 10B9F6, 5H8C12, A2.2/79, and C4.1/102) diluted 10^{-3} to 10^{-5} immediately before incubation. Inhibition controls were normal mouse serum (diluted 10^{-3}) and Alb4 (diluted 10^{-3}).

Inhibitions with cold Ag took place by mixing 5 ng radiolabeled *Der p 1*, *Der m 1*, or *Der f 1* in 50 μl with the same volume of partially purified *Der p 1*, *Der m 1*, or *Der f 1* (AML fractions) or the acid-denatured *Der p 1* immediately before incubation with immobilized mAb or patient IgE. The ranges of inhibitor concentrations used were 0.35 to 35,000 ng/ml native/denatured *Der p 1*, 1.0 to 100,000 ng/ml *Der m 1* and 0.47 to 47,000 ng/ml *Der f 1*. Control inhibitions with 1 to 1000 $\mu\text{g/ml}$ of allergenic pollen extracts (*Phleum pratense* and *Betula verrucosa*) gave maximal inhibitions of 15 to 20%.

Determination of association constants (K_a values). Radiolabeled Ag, *Der p 1*, *Der m 1*, and *Der f 1*, were affinity-purified on CNBr-activated Sepharose, coupled with mAb Nhp1, 4C1B8, and 4C1B8, respectively. Elution was performed with sodium carbonate buffer (pH 10) containing 50% ethyleneglycol, and followed by dialysis and freeze-drying. Eluted antigen produced dose-response curves parallel with those of standard antigens in our sandwich ELISA (20) and more than 95% of added counts were retained after incubation with PVC-immobilized monospecific rabbit antibodies.

Binding analysis was performed in the molar range 0.25 to 75×10^{-9} for mAb and 0.1 to 100×10^{-8} for labeled or labeled + cold Ag. Ag and mAb ($0.1 + 0.1 \text{ ml}$) was mixed in 2.5 ml polystyrene tubes and incubated overnight at 4°C after which 0.05 ml rabbit-anti

⁵ Wahn, U., C. Schweter, P. Lind, and H. Löwenstein. Prospective study of immunologic changes induced by two different *Dermatophagoides pteronyssinus* extracts, prepared from whole mite culture and mite bodies. Submitted for publication.

mouse IgG (DAKO, code Z-259), diluted 1/10, was added for another overnight incubation. All dilutions were done in PBS, containing 1 mg/ml BSA and 15 mM sodium azide. Normal mouse serum was added at 1/100 (v/v) for mAb concentrations below 5×10^{-9} . Centrifugation took place at $1000 \times g$ for 15 min and the precipitate was counted. No washings were performed and counts obtained for the control mAb, Fd-1, within the same range of Ag and antibody molarities were used as corrections of unspecific precipitation. The binding data were treated graphically according to Scatchard or by plotting reciprocal molarities of bound vs free Ag and calculating K_a as $1/(2 \times \text{slope} \times \text{known antibody molarity})$. The latter plot was restrictively used for experiments with maximal binding below 50%.

Sequence analysis. N-terminal amino acid sequence analysis was performed on a gas phase microsequencer, Applied Biosystems Inc. model 470A (27), using the program "02NRUN" supplied with the instrument. The released PTH amino acids were analyzed on a Waters HPLC system equipped with UV monitoring at 254 and 313 nm. The chromatographic separation of PTH amino acid derivatives and sequencer artefacts was achieved with a Superspher C8 column (125×4 mm) from Merck, F.R.G., and a gradient of 19% methanol to 38% ethanol in 0.01 M ammonium acetate, pH 3.9. Serine and threonine were partially detected as their dehydro forms which absorb at 313 nm. Cysteine was detected after reduction and derivatization to S-(pyridylethyl)-cysteine with 4-vinylpyridine (28). Each of the three proteins were analyzed at least twice, and 50 to 60 μ g of protein (corresponding to 2 nmol) was applied in each analysis. Repetitive yields were 86 to 92%.

RESULTS

Mutual inhibitions of labeled *Der p* 1 binding were carried out with a total of 19 immobilized mAb and 21 inhibitor mAb, including the *Der f* 1-specific 6A8B10 (8) and the control mAb, Alb4. Homologous inhibitions (same mAb on solid phase and in solution) generally exceeded 80% at dilutions below 10^{-4} . Large variations in the inhibitory potency of heterologous, inhibiting mAb were found in experiments with the same solid-phase mAb (Fig. 1). Control mAb and 6A8B10, exerted inhibition in some cases at large concentrations (10 μ g/ml) but this did not exceed 50%. The overall pattern (Table I and corresponding data from further six mAb) led to a classification in four groups, within each of which generally all mAb inhibit Ag binding of the others. Few significant inhibitions were obtained outside the four groups.

The binding of labeled antigens to selected mAb was

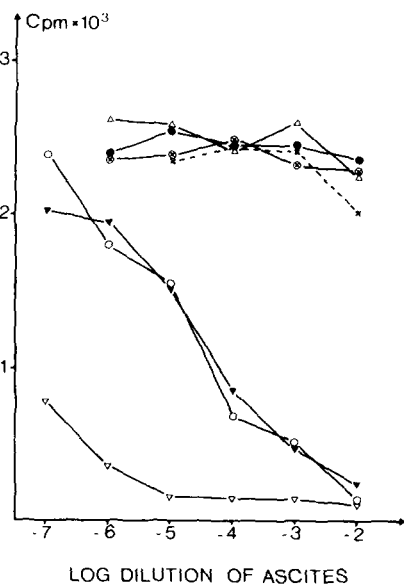


Figure 1. Example of inhibition of labeled *Der p* 1 binding to immobilized mAb. Nhp3 was coated (0.3 μ g/ml) onto PVC trays. Binding of ^{125} I-*Der p* 1 (5 ng, 29,800 cpm) was inhibited by mixing with 10^{-7} to 10^{-2} dilutions of raw ascites or ascites reconstituted at 10 mg/ml: ●, Nhp1; Δ, Nhp2; ▽, Nhp3; ○, Nhp4; ◻, Nhp5; ×, 6A8B10; ▲, A2.2/79.

studied in a solid-phase assay, by using immobilized mAb and in a liquid-phase assay using precipitation with second antibody. When association constants (K_a values), as determined by Scatchard analysis were high (above 10^8) a binding percentage above 10% in the solid-phase assay was observed for the same mAb/Ag combination. K_a values below 10^7 generally resulted in solid-phase binding percentages less than twice the level obtained with control mAb (Table III).

Labeled *Der p* 1 binding to immobilized mAb was inhibited by cold *Der p* 1, *Der m* 1, *Der f* 1, and acid-denatured *Der p* 1 by using inhibitor concentrations over a 5 log range (Fig. 2). The ratio between homologous and heterologous inhibitor potency was generally reflected by the ratio between the corresponding K_a values, although the latter tend to be numerically larger (cf. Table III).

Acid-denatured *Der p* 1 elicited none or negligible inhibition of binding to 14 mAb, produced 50 to 60% inhibition at 10^5 ng/ml for Nhp5, 10B9F6, and A2.2/79 and produced 60% inhibition at 10^4 ng/ml of the binding to C4.1/96 (Table III and data not shown).

For the 19 available mAb, the *Der p* 1/*Der f* 1 ratio of inhibitory potency was plotted against the *Der p* 1/*Der m* 1 ratio (Fig. 3). A spectrum of cross-reactivities existed within each of the four groups of mAb defined by relative binding position.

The N-terminal sequences (Table II) showed 93% (28 of 30) identity between *Der m* 1 and *Der f* 1 and 70 to 77% (21 of 30 to 23 of 30) identity between *Der p* 1 and *Der m* 1/*Der f* 1 in good accordance with morphologic taxonomy, where *D. farinae* and *D. microceras* are considered sibling species (4). Our data for *Der f* 1 are in 100% accordance with the N-terminal sequence data (20 steps) published recently by Heyman et al. (8).

Two distinct fractions of isolated *Der p* 1, differing by the conditions used for elution from phenyl-Sepharose (6), were sequenced and both fractions exhibited a heterogeneity at step 2 in the Edman degradations: Fraction 1 yielded PTH-Asn and PTH-Gln in varying ratios, whereas Fraction 2 consistently released PTH-Asn and PTH-Gln in a ratio of approximately 2 to 1. It is possible that this heterogeneity represents a true genetic dimorphism in our cultures. A conclusion regarding this will await sequence data of *Der p* 1 from other culture isolates.

The binding of labeled *Der p* 1 to immobilized rabbit antibodies and to two pools of IgE sera were inhibited by four dilutions of selected mAb to *Der p* 1 and the control mAb, Alb4. Maximal percentages inhibition elicited by the mAb ranged from 7 to 24% for binding to the rabbit antibodies and from 31 to 47% for binding to human IgE antibodies. The binding of labeled *Der p* 1 to immobilized IgE from 10 individual allergic patients were furthermore inhibited from 12 to 65% by 8 mAb (Table IV and data not shown).

The binding of labeled *Der p* 1 to immobilized IgE from 7 allergic adults was inhibited by cold *Der p* 1, *Der m* 1, *Der f* 1, *Der m* 1 + *Der f* 1 (a 2:1 mixture) and acid-denatured *Der p* 1 in a 5 log range of concentrations. Cold *Der p* 1 was the strongest inhibitor throughout, yielding 50% inhibition of binding generally at 10 to 100-fold lower concentrations than the most potent heterologous inhibitor and generally at 100–10,000 fold lower concentration than the weakest heterologous inhibitor (Fig. 4, Table V). For serum Br20, however, *Der m* 1 and *Der p* 1

TABLE II
 N-terminal amino-acid sequences of *Der f* I, *Der m* I, and *Der p* I^a

	5					10					15				
<i>Der f</i> I	Thr	Ser	Ala	Cys	Arg	Ile	Asn	Ser	Val	Asn	Val	Pro	Ser	Glu	Leu
<i>Der m</i> I	Thr	Gln	Ala	Cys	Arg	Ile	Asn	Ser	Gly	Asn	Val	Pro	Ser	Glu	Leu
<i>Der p</i> I	Thr	Asn	Ala	Cys	Ser	Ile	Asn	***	Gly	Asn	Ala	Pro	Ala	Glu	Ile
		Gln													
	20					25					30				
<i>Der f</i> I	-Asp	Leu	Arg	Ser	Leu	Arg	Thr	Val	Thr	Pro	Ile	-(Arg)	Met	Gln	Gly
<i>Der m</i> I	-Asp	Leu	Arg	Ser	Leu	Arg	Thr	Val	Thr	Pro	Ile	Arg	Met	Gln	Gly
<i>Der p</i> I	-Asp	Leu	Arg	Gln	Met	-(Arg)	Thr	Val	Thr	Pro	Ile	Arg	Met	Gln	Gly

^a Residues identical in at least two of the sequences are shown in bold face. Tentatively identified residues are shown in parentheses; *** indicates a one-residue gap introduced in the *Der p* I sequence to give maximal alignment with the other two sequences.

 TABLE III
 Antibody binding of labeled Ag and inhibition of labeled *Der p* I binding to immobilized mAb

	Nhp1	Nhp2	Nhp3	Nhp4	Nhp5	Nhp6	4C1B8	Fd1/Alb4
Binding (%) ^a								
<i>Der p</i> I	9.9	12.5	12.2	11.7	10.9	9.5	8.6	0.7/1.1
<i>Der m</i> I	3.4	1.4	9.5	2.0	1.1	2.6	9.6	1.6/2.1
<i>Der f</i> I	3.1	2.5	1.1	1.6	0.9	6.2	13.7	1.6/1.9
<i>K_a</i> values ^b (M ⁻¹)								
<i>Der p</i> I	2.4 × 10 ⁸	3.5 × 10 ⁸	2.5 × 10 ⁹	1.2 × 10 ⁹	1.3 × 10 ⁹	2.0 × 10 ⁹	1.5 × 10 ⁸	
<i>Der m</i> I	6.8 × 10 ⁵	<3 × 10 ⁵	1.9 × 10 ⁹	4.2 × 10 ⁶	<3 × 10 ⁵	1.0 × 10 ⁷	2.5 × 10 ⁸	
<i>Der f</i> I	6.3 × 10 ⁵	4.0 × 10 ⁵	<3 × 10 ⁵	2.8 × 10 ⁶	<3 × 10 ⁵	4.3 × 10 ⁸	4.8 × 10 ⁸	
Inhibition potency ^c (M ⁻¹)								
<i>Der p</i> I	7.0 × 10 ⁸	2.2 × 10 ⁸	8.0 × 10 ⁸	2.5 × 10 ⁹	1.8 × 10 ⁹	1.0 × 10 ⁹	1.1 × 10 ⁹	(2.8 × 10 ⁸) ^d
<i>Der m</i> I	7.0 × 10 ⁷	1.6 × 10 ⁶	1.6 × 10 ⁸	8.0 × 10 ⁷	<5 × 10 ⁵	1.1 × 10 ⁷	7.1 × 10 ⁹	(5.0 × 10 ⁸)
<i>Der f</i> I	2.2 × 10 ⁷	1.0 × 10 ⁷	2.0 × 10 ⁶	1.8 × 10 ⁷	1.8 × 10 ⁶	4.0 × 10 ⁸	3.2 × 10 ⁹	(6.3 × 10 ⁸)
Δ <i>Der p</i> I ^e	<1 × 10 ⁶	<1 × 10 ⁶	<1 × 10 ⁶	<1 × 10 ⁶	<1 × 10 ⁶	ND	<1 × 10 ⁶	(ND)

^a PVC trays were coated with mAb in concentrations from 0.01 to 10 μg/ml. Figures denote the percent binding of 5 ng radiolabeled antigens at optimal coating concentration (10 μg/ml, except Nhp3: 1 μg/ml).

^b Association constants determined by liquid phase binding.

^c PVC trays were coated with mAb (0.8 to 7.8 μg/ml, except Nhp3: 0.3 μg/ml). The binding of 5 ng radiolabeled *Der p* I was inhibited by 1 to 10⁵ ng/ml of cold Ag. Figures denote reciprocal molarities at 50% binding inhibition.

^d Figures in parentheses are inhibition of labeled *Der f* I binding to 4C1B8.

^e Acid-denatured *Der p* I.

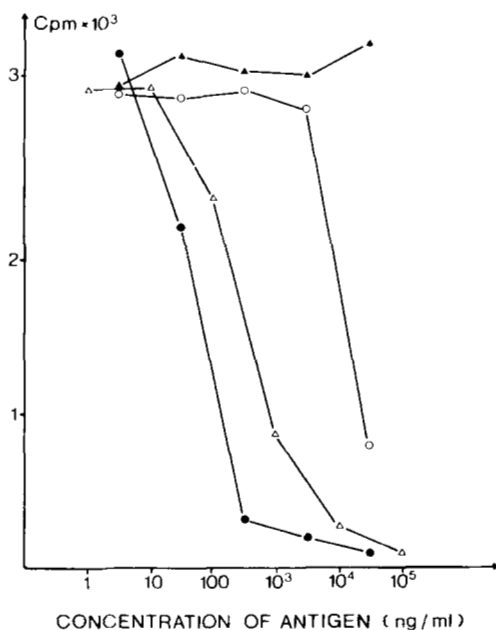


Figure 2. Example of inhibition of labeled *Der p* I binding to mAb by cold Ag. Immobilized (0.3 μg/ml) Nhp3 was incubated with 5 ng labeled *Der p* I in mixture with partially purified, cold *Der p* I (●), *Der m* I (Δ), *Der f* I (○), and with acid-denatured *Der p* I (▲). Retained cpm are plotted against inhibitor concentration as nanograms *Der p* I/*Der m* I/*Der f* I per milliliter.

were of approximately equal potency. These data indicate that most sera contained IgE with a large degree of species specificity to *Der p* I relative to *Der m* I and/or *Der f* I.

Four sera (Br4, 12, 17, and 20) were inhibited 50% by approximately equal concentrations of *Der m* I and of the

two-species mixture, *Der m* I + *Der f* I, whereas 10 to 100-fold larger concentrations of *Der f* I were required. This indicates the existence in these sera of IgE antibodies cross-reactive to *Der p* I and *Der m* I but distinct to *Der f* I. The species preferences present in the sera were classified according to the criteria in Table V in order to compare with results of a previous study (14), using column-absorbed IgE sera, radiolabeled anti-IgE and antibody-precipitated allergens (Table V, last two columns).

Acid-denatured *Der p* I produced inhibition curves parallel with those of native *Der p* I (Fig. 4), but approximately 500-fold larger concentrations were needed to affect 50% inhibition of labeled Ag binding.

DISCUSSION

Mutual inhibitions of labeled Ag binding demonstrated a clustering of the available mAb in four groups (Table I). The groups so defined are interpreted to represent each a region on the surface of the antigen with one or several epitopes sufficiently close to exclude simultaneous binding of two mAb. The symmetrical pattern of inhibition favors this interpretation, although effects via allosteric inhibition cannot be entirely excluded. It was not surprising to find a considerable overlap between two regions. Similar patterns have been observed for other strong immunogens, such as BSA, hen egg white lysozyme, and influenza virus hemagglutinin (reviewed in Ref. 19).

Experimentally, the concentrations needed to elicit 50% inhibition of labeled Ag binding would depend on the coating concentration of the immobilized mAb and differences in binding affinity. Indications of this is seen

Figure 3. Spectrum of species specificities of 19 mAb as calculated from inhibitions of labeled *Der p* I binding with cold antigens. Horizontal axis, Difference between $\log C_{50\%inh}$ for *Der m* I and *Der p* I. Vertical axis, Difference between $\log C_{50\%inh}$ for *Der f* I and *Der p* I. mAb belonging to the same inhibitory group are encircled.

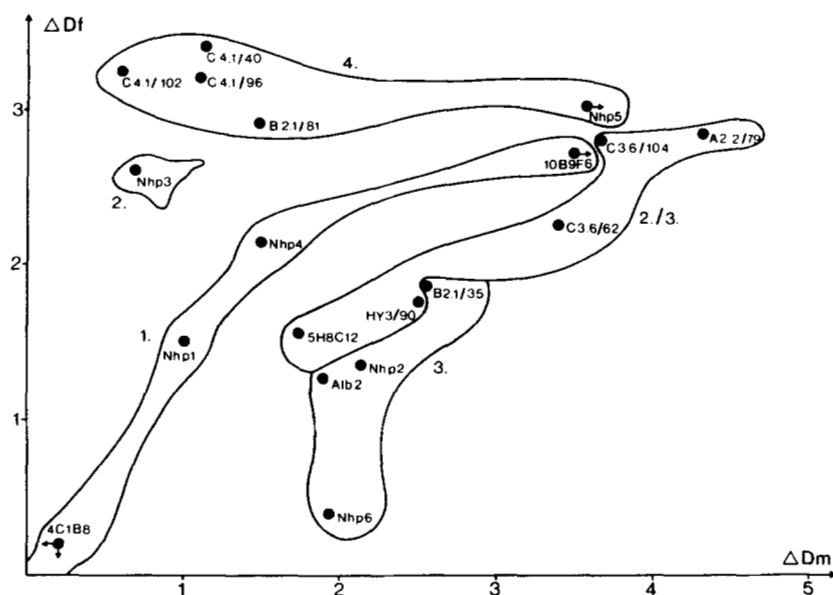


TABLE IV
mAb inhibition of labeled *Der p* I binding to immobilized rabbit IgG or human IgE antibodies^a

	Cpm (0) ^b	Nhp4	Nhp3	Nhp2	Nhp5
R1a <i>Der p</i> I	5832	14	24 ^c	16 ^c	15 ^c
R2a <i>Der p</i> I	6522	9	7 ^c	9	12 ^c
Br-pool	1150	39 ^c	47 ^c	32	45 ^c
UW-pool	1771	39 ^c	41 ^c	34	39 ^c
Br4	1636	39	52	32	42
Br5	1270	31 (31)	23 (7)	29 (0)	29 (8)
Br9	1346	24 (11)	49 (21)	38 (17)	41 (19)
Br12	1435	41 (13)	47 (33)	19 (0)	37 (32)
Br17	1080	50	53	25	39
Br20	809	27	65	50	33
Br25	1526	59	28	28	30
UW12	1658	35	35	25	46
UW17	1946	27	31	39	47
UW46	1916	46	50	35	24

^a Antibodies from two rabbits were coated to PVC trays at 5 μ g/ml. Pooled or individual human IgE sera (diluted 1/2 to 1/10) were immobilized by reaction with rabbit anti-human IgE coated at 5 μ g/ml to PVC trays. Figures denote the percent inhibition of labeled *Der p* I (5 ng, 39,500 cpm) binding when mixed with ascites diluted 10^{-3} . Inhibitions obtained with overnight incubation of labeled Ag + inhibitor shown in parentheses.

^b Counts obtained without added inhibitor. The levels of unspecific binding (obtained with 35,000 ng/ml cold *Der p* I as inhibitor) were 65 to 102 cpm. Normal human serum bound 126 cpm.

^c Saturation obtained at ascites dilution 10^{-4} .

when, e.g., inhibitions of 4C1B8, Nhp1, and Nhp4 are compared. The 5 to 10 times larger affinity of the latter (Table III) is probably responsible for its larger inhibitory potency (Table I, upper left). However, with the chosen experimental design, these variations are not likely to affect the grouping as such, because specific inhibitions could be recorded in large excesses of inhibitor relative to labelled antigen and because the results of two reverse inhibitions were used for each pair of mAb.

The 19 mAb employed in the present study originated from four laboratories using a variety of immunization and fusion regimens and different screening assays. Each of the four groups detected contained representation of mAb from at least two laboratories, so the regions of the Ag thus defined probably constitute the immunodominant parts of the molecule, at least in the BALB/c mouse.

Similar patterns of species specificity were obtained,

whether investigated by direct solid or liquid phase binding assays with labeled Ag of three species (*Der p* I, *Der m* I, and *Der f* I) or assayed by inhibition of labeled *Der p* I binding by the same set of cold Ag (Table III). The determination of association constants yields direct measures of relative binding affinity. For studies of large arrays of mAb we prefer the inhibitory assay, due to its technical simplicity and because no bias from possible damage of binding sites by the labeling process (12) is introduced.

It is notable that mAb directed against the same or closely positioned sites on the antigen surface were found to have very different degree of species specificity (Fig. 3). As an example, mAb of group four all displayed low activity towards *Der f* I, but whereas Nhp5 was highly species specific, four other clones in the same group cross-reacted with *Der m* I.

The Ag of the three species are physicochemically very similar with molecular weights of 25 to 30 kDa by SDS-PAGE (7, 8). The frequent occurrence of *Der p* I specific mAb is not surprising, when related to the available part of the amino acid sequences (Table II). In the first 30 residues of the sequence *Der p* I differed from *Der m* I and *Der f* I in 7 and 9 positions, respectively, counting the gap at position 8 as an amino-acid change. The latter two Ag diverged at positions 2 and 9, and it is interesting that *Der m* I sustain identity with *Der p* I in at least position 9 (Table II). Relations of this type provide an explanation for antibody specificities like the *Der p* I/*Der m* I cross-reactivity of Nhp3 and other mAb. These occurred more frequent than anticipated from the phylogenetic relationship: *D. pteronyssinus* is easily distinguished from *D. microceras* and *D. farinae*, which are sibling species, with only minute morphologic differences.

The relative binding positions and the species specificity of mAb obtained in the present study are in accordance with previously performed enzyme immunoassays of mAb Nhp1-Nhp6 (12), where a different assay design, employing immobilized Ag, was used.

Selected mAb exerted strong inhibition of labeled Ag binding to human IgE (Table IV). Uninhibited levels of

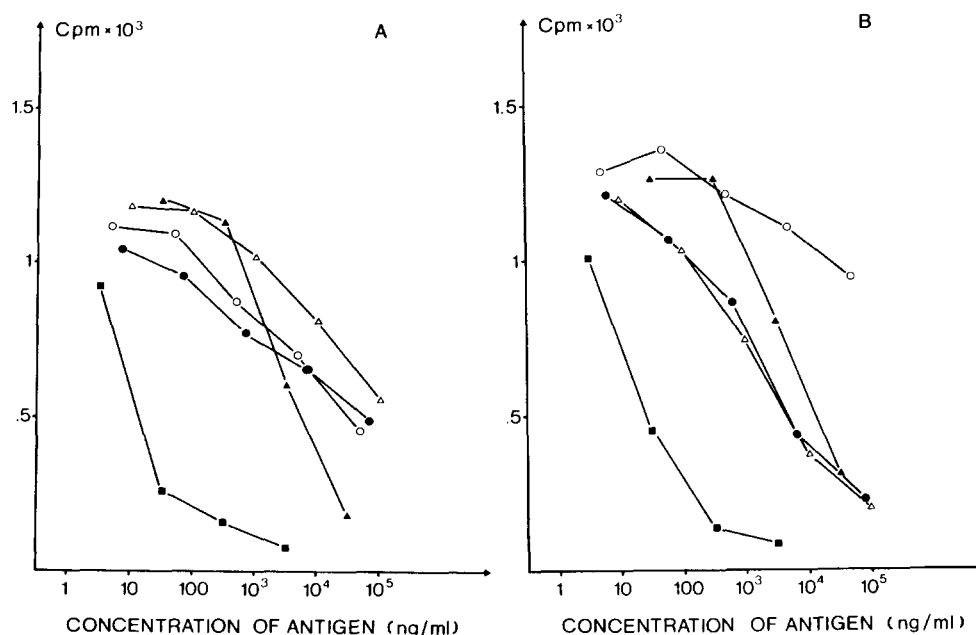


Figure 4. Inhibition of labeled *Der p* I binding to immobilized human IgE with cold Ag. A. Serum Br9; B. Serum Br12. Patient serum (100 μ l, diluted 1/5) was added to PVC trays coated with rabbit anti-human IgE (5 μ g/ml). Labeled *Der p* I (5 ng) was added in mixture with cold *Der p* I (■), *Der m* I (Δ), *Der f* I ($\circ$), *Der m* I + *Der f* I (2:1 w/w) (●) and with acid-denatured *Der p* I ($\blacktriangle$). Retained counts are plotted against inhibitor concentrations in nanograms *Der p* I/*Der m* I/*Der f* I per milliliter.

TABLE V
Inhibition of labeled *Der p* I binding to seven human IgE sera by cold Ag^a

	<i>Der p</i> I	<i>Der m</i> I	<i>Der f</i> I	<i>Der m</i> I + <i>Der f</i> I	Denatured <i>Der p</i> I	Specificities ^b	
						This study	Previous study ^c
Br4	1.05 (99%)	2.10 (93%)	3.50 (69%)	2.10 (93%)	3.60 (82%)	p, pm	(p), (pm), (pf)
Br5	0.50 (100%)	2.30 (88%)	1.65 (87%)	1.80 (89%)	3.00 (95%)	p	p, (pf)
Br9	0.75 (100%)	4.20 (65%)	3.30 (72%)	2.95 (70%)	3.20 (93%)	p, pf	p, pf
Br12	1.00 (100%)	3.00 (91%)	>5.00 (34%)	3.15 (89%)	3.65 (82%)	p, pm	p, pm
Br17	0.85 (100%)	2.60 (82%)	4.55 (53%)	2.70 (84%)	3.50 (77%)	p, pm	p, (pm)
Br20	0.75 (95%)	0.85 (100%)	3.65 (76%)	0.95 (100%)	3.55 (80%)	pm	(pm)
Br25	0.55 (99%)	1.45 (89%)	1.65 (90%)	1.15 (92%)	3.25 (87%)		p, (pf)

^a IgE from individual patient sera were immobilized on PVC trays, coated with rabbit anti-human IgE. The binding of labeled *Der p* I (5 ng) was inhibited by cold Ag in concentrations from 1 to 10⁵ ng/ml. Figures denote the log concentrations at 50% inhibition of binding. Figures in brackets are the maximal percentages inhibition obtained.

^b Criteria for specificity classification: $C_{50\%inh}(Dm + Df) - C_{50\%inh}(Dp) > 1.0$, p; $C_{50\%inh}(Df) - C_{50\%inh}(Dm + Df) > 1.0$, pm; and $C_{50\%inh}(Dm) - C_{50\%inh}(Dm + Df) > 1.0$, pf.

^c Classified according to column absorption of serum and subsequent binding to gel-precipitated Ag and detection by radiolabeled rabbit anti-human IgE and autoradiography according to Lind et al. (14).

bound Ag were, however, low: 0.1 to 0.2 ng, and large excess of mAb (200 to 500 ng) was needed to obtain saturation of inhibition. One explanation may be superior affinities between antigen and populations of IgE as compared to mAb. Alternatively, sterical factors may play a greater role in the binding of antigen to IgE in the employed three-layer assay, because Ag attachment to two or more IgE molecules (of different epitope-specificity) may enhance retention on the solid-phase during subsequent washing. Indication of the latter possibility was obtained by prolonging the incubation period for labeled Ag + inhibitor, in which case some (but not all) mAb had their inhibitory capacity markedly reduced (Table IV). Inhibitions with Fab fragments were not feasible at the

time the experiments were performed. However, the obtained data demonstrate, that the binding sites for IgE are distributed over the molecular surface, coinciding to a significant degree with the binding areas for mouse mAb.

Only slight differences were observed regarding the inhibitory potency of the acid-denatured *Der p* I toward Ag binding to mAb and to human IgE. Acid-denaturation produced more variable reductions of the inhibitory potency toward the mAb (range 2 to 4 log) than toward individual IgE sera (2.45 to 2.80 log). However, neither the investigated mAb nor the human IgE antibodies appeared to be directed against sequential epitopes. The inhibitory effect may be simplest interpreted as caused

by a small residual fraction of native antigen molecules in the denatured preparation.

The spectrum of species specificities detected in the panel of mAb and in the human IgE sera were similar. The differences between concentrations of homologous and heterologous inhibitors needed to elicit 50% inhibition of labeled Ag binding were in the same range (1 to 4 log) for mAb and IgE, and the presence of antibodies of *Der p 1* specificity and two-species cross-reactivity were detected for both types of antibodies.

Our conclusion in this regard is at variance with that of Heyman et al. (8), whose data were interpreted so as to reveal a dichotomy between the IgG response of mice and the IgE response in humans with respect to epitope specificity on *Der p 1* and *Der f 1*. However, their comparison of species specificities present in the two types of antibodies was based on a direct antigen binding assay for the mouse mAb and on a solid-phase absorption technique for human IgE and mouse IgG. In the present study the species specificity of mAb and IgE was assessed in directly comparable designs: inhibition of labeled Ag binding to immobilized antibodies, and no essential differences were found. Furthermore, the specificities of IgE found in the present study corresponded well with those found by immunoabsorption in a previous study on the same patient material (14).

An interrelation between relative binding location on the antigen and species specificity could not be performed on the human IgE antibodies, because of their polyclonal nature. Thus, it is not at present possible to state, whether there are at least three physically distinct, species-specific epitopes on *Der p 1* for IgE, as was found for the mAb (Fig. 3). However, the extensive analogy between the spectra of cross-reactivity and the demonstrated overlap between binding regions for human IgE and mAb do not support the existence of a peculiar regulation of the epitope specificity of the human IgE response. Studies of the fine specificity of human IgE and IgG antibodies are planned employing anti-idiotypic reagent antibodies, raised against the mAb.

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