

RECOGNITION OF TWO *Dermatophagoides pteronyssinus*-SPECIFIC EPITOPES ON ANTIGEN P₁ BY USING MONOCLONAL ANTIBODIES: BINDING TO EACH EPITOPE CAN BE INHIBITED BY SERUM FROM DUST MITE-ALLERGIC PATIENTS¹

MARTIN D. CHAPMAN,^{2*} WILLIAM M. SUTHERLAND,[†] AND THOMAS A. E. PLATTS-MILLS*

From the *Division of Allergy and Clinical Immunology, Department of Medicine, and the †Department of Anatomy, University of Virginia, Charlottesville, VA 22908

Monoclonal antibodies were raised against Antigen P₁, the major allergen of the house dust mite (*Dermatophagoides pteronyssinus*). The majority were Antigen P₁ specific, isotype IgG1, and did not react with a comparable *D. farinae* allergen. These antibodies bound 38 to 50% of ¹²⁵I Antigen P₁ in antigen-binding assays (titer ≥1/1,000,000), and the quantities of IgG antibody in ascites were 2 to 4 logs greater than those in polyclonal mouse antiserum or in serum from a mite-allergic patient. Two IgM antibodies showed weak binding to Antigen P₁ but reacted strongly with *D. pteronyssinus* in enzyme immunoassay (titer ≥1/100,000). Assessments of the specificity of the IgG antibodies by using two inhibition radioimmunoassays suggested that they were directed against two different epitopes. Antibodies 10B9 F6 and 5H8 C12 were purified by preparative isoelectric focusing (isoelectric points of pI 6.25 and 7.4, respectively) and radiolabeled with ¹²⁵I. Cross-inhibition experiments, using ascites dilutions to inhibit binding of each radiolabeled antibody to Antigen P₁, confirmed that these antibodies recognized two distinct epitopes. Analysis of antibodies from 39 clones/hybrids showed that the majority were directed against the same epitopes as either 10B9 F6 or 5H8 C12 (3 out of 39 [8%] and 29 out of 39 [74%], respectively). None of the monoclonal antibodies significantly inhibited (>10%) human IgE binding to Antigen P₁ in the radioallergosorbent test. However, 12 of 14 sera from mite allergic patients inhibited binding by the monoclonal antibodies. One serum from a mite-allergic patient inhibited binding of both 10B9 F6 and 5H8 C12 by >85% and showed parallel inhibition curves. The results suggest that these monoclonal antibodies could be used to assay Antigen P₁ in both *D. pteronyssinus* and house dust extracts. It should also be possible to use monoclonal antibodies in inhibition assays to define the antigenic/allergenic deter-

minants recognized by human IgG and IgE antibodies on this mite allergen.

Allergens derived from the house dust mite, *Dermatophagoides pteronyssinus*, are an important cause of immediate hypersensitivity (1, 2). The major allergen of *D. pteronyssinus*, Antigen P₁ has been purified from extracts of mite culture and is a heat-labile glycoprotein, m.w. 24,000, which occurs in multiple isoallergenic forms (3). This allergen may be present in large quantities in house dust (up to 100,000 ng/g), and the main source of Antigen P₁ in both mite culture and house dust is mite feces (4, 5). Antigen P₁ has been purified in several laboratories (6–9) by using different purification techniques and different sources of mites and mite culture media. Positive immediate skin tests and serum IgG and IgE antibodies to Antigen P₁ have been demonstrated in mite allergic patients with rhinitis, asthma, and atopic dermatitis (10, 11). Proliferation of T cells in response to Antigen P₁ was recently reported (12). Furthermore, *in vivo* delayed in time eczematous skin lesions infiltrated with eosinophils and basophils can be induced with purified Antigen P₁ (13, 14). The antigenic/allergenic determinants on Antigen P₁ that elicit these immediate and delayed reactions have not been defined. Thus, it is not known whether human antibody responses to Antigen P₁ are restricted in specificity or polyclonal, whether IgG and IgE antibodies recognize the same or different antigenic determinants, or whether these determinants are the same as those that stimulate T cells.

With the advent of techniques for the production of monoclonal antibodies of single specificity (15), it has become possible to investigate antigenic determinants (or epitopes) on a variety of macromolecules, including allergens. There have been several recent reports of the production of monoclonal antibodies to antigenic components and allergens of rye grass pollen (16, 17), ragweed (18), wheat flour (19), and *Alternaria* (20). Monoclonal antibodies to dust mite have also been produced (21), and Aalberse (22) has produced antibodies that react with Antigen P₁, but detailed analysis of their epitope specificity has not been reported. We have produced a series of monoclonal antibodies to Antigen P₁ as part of further studies on the antigenic structure of this glycoprotein. These antibodies are *D. pteronyssinus* specific and do not bind to the analogous allergen from *D. farinae*. Furthermore, they define at least two distinct epitopes on

Received for publication April 16, 1984.

Accepted for publication June 25, 1984.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by National Institutes of Health Grant AI20565.

² To whom correspondence and reprint requests should be addressed. Present address: Division of Clinical Immunology-Allergy, Department of Medicine, UCLA School of Medicine, Center for Health Sciences, Los Angeles, CA 90024.

Antigen P₁, and binding to both epitopes can be inhibited by serum from mite-allergic patients.

MATERIALS AND METHODS

Antigens. Antigen P₁ was prepared from whole *D. pteronyssinus* culture (Bencard, Betchworth, Surrey, UK) by salt precipitation (50% saturated ammonium sulfate), gel filtration, and Pevikon block electrophoresis by using modifications of methods described (3, 23). The preparation used in these studies contained ~90% Antigen P₁ by comparisons of dry weight with Antigen P₁ content determined by inhibition radioimmunoassay. *D. farinae* allergen, Fraction I (*D. farinae* Fr. I),³ was extracted from *D. farinae* culture (Hollister-Stier, Spokane, WA) that had been depleted of mite bodies and was partially purified by salt precipitation and gel filtration. *D. farinae* Fr. I showed extensive cross-reactivity with Antigen P₁ on immunodiffusion and radioimmunoassay. Full details of the purification of this allergen will be published elsewhere (Heymann *et al.*, manuscript in preparation).

Immunization of mice and production of monoclonal antibodies to Antigen P₁. Ten BALB/c mice were immunized i.p. with 10 µg Antigen P₁ in complete Freund's adjuvant (CFA) (Difco, Detroit, MI) and were boosted twice with 7 µg Antigen P₁ in CFA at intervals of 10 days. Twelve days after the last injection, serum from each mouse was assessed for antibody production by enzyme-linked immunosorbent assay (ELISA). Seven of the 10 mice showed good antibody responses, and a mouse with an antibody titer of 1:12,800 was boosted i.v. with 25 µg Antigen P₁. Four days later, spleen cells from this mouse were washed once (200 × G for 10 min) with RPMI 1640 medium containing glutamine (GIBCO, New York, NY) enriched with 6 ml/liter MEM nonessential amino acids (100X; GIBCO), 10% NCTC-109 medium (M. A. Bioproducts, Walkersville, MD), 11 mM sodium bicarbonate, 1 mM sodium pyruvate, 0.06 mM β-mercaptoethanol, 50,000 U/liter penicillin and 50 mg/liter streptomycin. They were then mixed in an 8:1 ratio with Sp2/0-Ag 14 myeloma cells (24) (5 × 10⁶) spleen cells:6.2 × 10⁷ Sp2/0 myeloma cells) and centrifuged (200 × G for 5 min). The supernatant was aspirated, leaving "dry" cell sediments, which was resuspended in its trapped medium. Cells were fused by the stepwise addition of 1 ml 37% polyethylene glycol (PEG 1000, Koch-Light, New York) over 1 min (~0.2 ml/10 sec). Enriched RPMI 1640 (10 ml) was added dropwise over ~5 minutes, and the cells were gently resuspended and washed once in medium supplemented with 15% fetal bovine serum 6.7 mM hypoxanthine and 0.032 mM thymidine (HT medium). The cells were resuspended, transferred to a petri dish, and incubated at 37°C in a humidified atmosphere containing 5% CO₂ and air for 1 hr, and then resuspended in 100 ml/HT medium. This cell suspension was plated into ten 96-well tissue culture plates (Costar, Cambridge, MA) at ~5 × 10⁵ cells/well and fed 24 hr later with 100 µl/well HT medium containing 5 × 10⁻⁵ mM aminopterin (HAT). Cultures were fed 100 µl HAT medium at successive 2-day intervals for 6 days. Macroscopic growth of cells was evident from day 10 onward, and the cells were screened for antibody production 10 to 17 days post-fusion by ELISA. Antibody-producing hybrids were reassayed by antigen-binding radioimmunoassay by using ¹²⁵I-labeled Antigen P₁ and were selectively expanded into 2-ml cultures in HT medium. These hybrids were cloned by limiting dilution by using medium containing 20% fetal bovine serum selected for high cloning efficiency in the absence of feeder cells. (Lot No. 100398; HyClone, Logan, UT). Clones showing antibody production between 14 and 21 days were expanded into 25-cm² and 75-cm² flasks to obtain antibody-containing supernatants and to culture sufficient cells for cryopreservation and for the production of ascites fluids. Ascites was produced by injecting 0.2 to 1.0 × 10⁷ cells/mouse i.p. into BALB/c mice that had been injected i.p. with 0.5 ml Pristane (2,6,10,14 tetramethylpentadecane; Sigma, St. Louis, MO) 1 to 3 weeks previously. The isotype of each monoclonal antibody was determined by immunodiffusion by using either concentrated tissue culture supernatants (15×) or diluted (1/100) ascites against rabbit anti-mouse subclass antisera (Nordic, El Toro, CA).

ELISA for screening monoclonal antibodies. Microtiter plates (Dynatech, Immulon II, Alexandria, VA) were coated with ~1 µg/well of a 50% saturated ammonium sulfate fraction of whole *D. pteronyssinus* culture (*D. pteronyssinus* (50% SAS cut)) in 0.1 M sodium

carbonate buffer, pH 9.6, overnight at 4°C. The plates were incubated for 2 hr with tissue culture supernatant, dilutions of mouse antiserum to antigen P₁, or normal mouse serum, and then incubated for 2 hr with 0.1 ml of a 1/3,000 dilution of peroxidase-conjugated, affinity-purified, goat anti-mouse IgG (γ and light chain specific) (Code 6450; Tago, Burlingame, CA). Plates were washed five times with phosphate-buffered saline, pH 7.4, containing 0.5% Tween 20 (Sigma, St. Louis, MO) (PBS-T) between incubations. Bound enzyme-labeled antibody was detected by the addition of 100 µl 2,2'-azino-di(3-ethylbenzthiazoline sulfonic acid) (ABTS) (55 mg/100 ml) in 0.1 M citrate/phosphate buffer, pH 4.2, containing 0.03% H₂O₂. The absorbance at 414 nm (A₄₁₄) was read after 20 to 30 min in a microplate spectrophotometer (Multiskan; Flow, McLean, VA).

Antigen-binding radioimmunoassay by using ¹²⁵I allergens. This assay was modified from previously described antigen-binding techniques for measuring human IgG and IgE antibodies to Antigen P₁ (10, 11). Essentially, 100 µl monoclonal antibody (tissue culture supernate or dilutions of ascites) or polyclonal mouse antiserum to Antigen P₁ was incubated for 4 hr with 100 µl of a 1/100 dilution of ¹²⁵I labeled allergen (2.5 ng, specific activity 17 to 40 µCi/µg) in 100 µl of a 1/50 dilution of normal mouse serum. IgG antibody antigen complexes were precipitated overnight at 4°C by the addition of 0.1 ml of a 1/12 dilution of rabbit anti-mouse IgG (specific for γ and light chains). The precipitates were washed three times with borate-buffered saline, pH 8.0, and the residual radioactivity in the precipitate was counted in a gamma counter (4/200 Micromedic, Horsham, PA). Both mite allergens were radiolabeled by using a modified chloramine-T technique (10).

Preparative isoelectric focusing (IEF) of monoclonal antibodies 10B9 F6 and 5H8 C12. Preparative IEF in a flat bed of granulated gel (Ultradex; LKB, Gaithersburg, MD) was carried out by using the LKB Multiphor. Ascites (4 ml 10B9 F6 or 2.5 ml 5H8 C12) was mixed into a gel slurry containing 4 g Ultradex and 4% v/v carrier ampholytes, pH 5 to 8, (Pharmacia, Piscataway, NJ) in 100 ml distilled water and focused at 8 W for 16 hr at 10°C (3). Gel segments (0.7 cm) were eluted with 2 ml distilled water and assayed for pH, protein content (absorbance at 280 nm-A₂₈₀) and mouse IgG antibody to Antigen P₁. The IEF purification yielded 10.2 mg 10B9 F6 and 7.8 mg 5H8 C12.

Solid-phase inhibition radioimmune binding assays to assess the epitope specificity of monoclonal antibodies: i) Binding of ¹²⁵I Antigen P₁ to solid-phase monoclonal antibody. Flexible polyvinyl chloride microtiter plates (Costar) were coated overnight at 4°C with 100 µl of either a 1/10,000 dilution of 10B9 F6 ascites or a 1/100 dilution of 3H8 G8 or 5H8 C12 ascites in 0.1 M sodium carbonate buffer, pH 9.6. The coated plates were washed twice in PBS-T and then incubated for 1 hr with 1% BSA-PBS-T. After two additional washes, the plates were air dried and then incubated for 4 hr with 50 µl of dilutions of ascites together with 50 µl ¹²⁵I labeled Antigen P₁ (10 ng; ~80,000 to 90,000 cpm). The plates were washed five times with PBS-T, then air-dried, and the radioactivity bound to individual wells was counted. The diluent used for all solid-phase assays was 1% BSA-PBS-T.

ii) Binding of ¹²⁵I labeled monoclonal antibodies to solid-phase Antigen P₁. Microtiter plates were coated with 10 µg/well *D. pteronyssinus* (50% SAS cut) (equivalent to ~7 µg/ml/well Antigen P₁), then were incubated for 4 hr with 50 µl serial 10-fold dilutions of ascites (10⁻² to 10⁻⁵) together with either 50 µl ¹²⁵I-labeled 10B9 F6 or 50 µl ¹²⁵I-labeled 5H8 C12 (3 ng; 90,000 to 120,000 cpm). The plates were washed five times in PBS-T, and the ¹²⁵I antibody bound to each well was counted. All assays were performed in duplicate. Sera from 14 patients with positive skin tests and serum IgG and IgE antibodies to *D. pteronyssinus* were compared in inhibition experiments with sera from four nonallergic, i.e., skin test-negative, controls. The percent specific inhibition of monoclonal antibody binding was calculated as follows:

% Specific inhibition

$$= \frac{\% \text{ inhibition by test serum} - \% \text{ inhibition control sera}}{100 - \% \text{ inhibition control sera}} \times 100$$

Inhibition of the radioallergosorbent test (RAST) by using monoclonal antibodies. Ascites dilutions (10⁻² or 10⁻⁴) or PBS controls were incubated overnight with cyanogen bromide-activated paper discs that had been coupled with *D. pteronyssinus* (50% SAS cut). Sera from six mite-allergic patients were diluted from 1/2 to 1/10 and assayed in RAST by using these discs as described (10). All of the sera showed strong RAST binding (5 to 10% binding of ¹²⁵I anti-IgE) and the quantities of IgG and IgE binding activity for Antigen P₁ in these sera ranged from 166 to 1200 U/ml and 45 to 400 U/ml, respectively.

Control monoclonal antibodies. Two monoclonal antibodies, 8G2

³ Abbreviations used in this paper: 1% BSA PBS-T, 1% bovine serum albumin in phosphate-buffered saline, pH 7.4, containing 0.25% Tween 20; *D. pteronyssinus* (50% SAS cut), 50% saturated ammonium sulfate fraction of whole *Dermatophagoides pteronyssinus* culture; ELISA, enzyme-linked immunosorbent assay; *D. farinae* Fr. I, partially purified Fraction I of *D. farinae*; IEF, isoelectrofocusing; RAST, radioallergosorbent technique.

B9 (IgG2a) and 10H11 D9 (IgG2a), which had been raised against *Trypanosoma cruzi* (25) were used as controls. Antibody 8G2 B9 had been shown to react with a specific *T. cruzi* glycoprotein by radioimmunoassay and by electrophoretic (25).

RESULTS

A series of monoclonal antibodies was derived from a single fusion by using spleen cells from a mouse that had been immunized with Antigen P₁. On screening, supernatants from 88 hybrids gave positive anti-*D. pteronyssinus* reactions in ELISA, and 70 of these (~7% of the total hybrids) had IgG binding activity for Antigen P₁. Positive binding ranged from 2- to 35-fold above the background for control monoclonal antibodies (≤ 250 cpm), and strongly positive supernatants could bind up to 40% of the added ^{125} I Antigen P₁. In contrast, none of 43 supernatants tested had detectable binding activity for ^{125} I *D. farinae* Fr. I. Twenty-seven clones secreting monoclonal antibodies to *D. pteronyssinus* were established from 16 parental hybrids. The properties of 11 of these antibodies are summarized in Table I: eight were isotype IgG1, one was IgG2a, and two were IgM. Ascites fluid containing IgG antibodies bound strongly to Antigen P₁ but failed to bind *D. farinae* Fr. I. Polyclonal mouse antiserum to Antigen P₁ also showed poor binding to *D. farinae* Fr. I (Table I). By contrast, mouse anti-*D. farinae* Fr. I antiserum bound almost equally to both mite allergens. The two IgM antibodies, 7E3 E8 and 7E10 F6, gave strongly positive anti-*D. pteronyssinus* reactions in ELISA (titer $\geq 1/100,000$) but showed weak binding to Antigen P₁. These IgM antibodies appeared to be *D. pteronyssinus* specific because they did not react with *D. farinae* in ELISA (data not shown).

The antigen-binding curves of five monoclonal IgG

TABLE I
Species specificity and isotypes of monoclonal antibodies to *D. pteronyssinus* Antigen P₁

Monoclonal Antibody	Isotype	ELISA (A414) ^a	^{125} I Antigen P ₁ Bound (cpm) ^b	^{125} I <i>D. farinae</i> Fr. I Bound (cpm) ^b
10B9 F6	IgG1	0.78	17,346	124
10B9 B6	IgG1	0.73	17,445	89
10H2 C6	IgG1	0.86	19,594	95
3H8 G8	IgG1	0.53	13,792	100
5H8 C12	IgG2a	1.20	14,237	84
2G7 E8	IgG1	0.45	8,688	81
5B1 C12	IgG1	0.59	11,190	80
3A6 A12	IgG1	0.57	14,846	132
5H8 D8	IgG1	0.50	5,792	82
7E3 E8	IgM	0.91	684	ND
7E10 F6	IgM	0.80	723	ND
Controls				
8G2 B9 ^c	IgG2a	0.17	322	89
10H11 D9 ^c	IgG2a	0.18	125	73
Mouse antiserum to Antigen P ₁ ^d	Polyclonal	1.34	21,981	1,362
Mouse antiserum to <i>D. farinae</i> Fr. I ^e	Polyclonal	ND ^f	5,352	4,953
Normal mouse serum	—	0.31	145	95

^a A₄₁₄ values for undiluted tissue culture supernatant assayed on microtiter plates coated with *D. pteronyssinus* (50% SAS cut).

^b Antigen-binding assays on monoclonal antibody ascites (10^{-4} dilution) or polyclonal mouse antisera (10^{-2} dilution). Values are mean of duplicate tubes. Total radioactivity added = 37,755 cpm for ^{125} I antigen P₁ and 38,252 cpm for ^{125} I *D. farinae* Fr. I.

^c Control monoclonal antibodies raised against *Trypanosoma cruzi* (25).

^d Pooled antiserum from eight BALB/c mice immunized with Antigen P₁ in CFA.

^e Antiserum from a mouse immunized with *D. farinae* Fr. I in CFA. Quantity of ^{125} I *D. farinae* Fr. I bound by IgG antibody in this antiserum, is lower because allergen preparation was not fully purified.

^f ND = not determined.

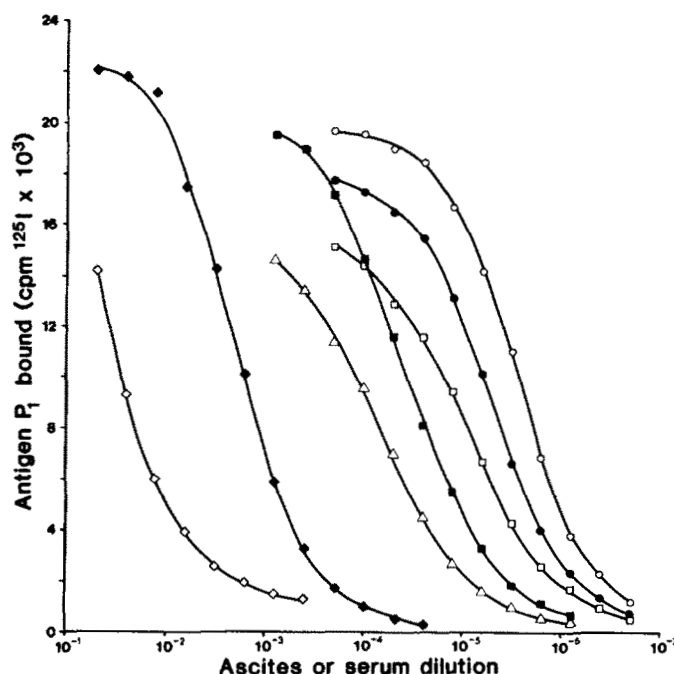


Figure 1. Antigen-binding curves of monoclonal antibodies to Antigen P₁. The binding of IgG ascites from clones 10B9 F6 (●), 10H2 C6 (○), 5H8 C12 (■), 3A6 A12 (□), and 2G7 E8 (△) to ^{125} I Antigen P₁ was compared with that of IgG antibody in polyclonal mouse antiserum to Antigen P₁ (◆; Table I) and in human serum from a mite-allergic patient (◇). The human serum contained 1000 U/ml IgG antibody to Antigen P₁ (10, 11). Total ^{125} I Antigen P₁ added = 38,000 cpm.

antibodies were compared with those of polyclonal mouse antiserum to Antigen P₁ and human serum from a mite-allergic patient (Fig. 1). The monoclonal antibodies showed positive binding of Antigen P₁ at dilutions of $\geq 1/1,000,000$, and maximal binding ranged between 38 and 52% of the added radioactivity. Binding of these five monoclonal antibodies was studied over a range of antigen concentrations from 450 to 1.5 ng/ml. Analysis of the binding curves showed ED₅₀ values ranging from 4.1×10^{-9} to 8.6×10^{-8} . Full Scatchard analysis showed K₀ values of 7×10^9 liters/mol for 10B9 F6 and 1.4×10^9 liters/mol for 5H8 C12. The binding curves of three monoclonal antibodies—10B9 F6, 10H2 C6, and 5H8 C12—closely paralleled those for IgG antibody in polyclonal mouse antiserum and in human serum, although the quantities of antibody in these sera were two to four orders of magnitude lower than in the ascites.

Epitope specificity of monoclonal antibodies. Two types of inhibition radioimmune binding assay were used to determine whether the monoclonal antibodies were directed against the same or different epitopes on Antigen P₁. Initially, the binding of ^{125} I Antigen P₁ to a solid-phase monoclonal antibody (10B9 F6, 3H8 G8, or 5H8 C12) was inhibited by using either homologous or heterologous ascites (Table II). Two groups of monoclonal antibodies could be distinguished by inhibition in these assays. One group completely inhibited the binding of Antigen P₁ to 10B9 F6 but did not inhibit the binding of this allergen to either 3H8 G8 or 5H8 C12 more than control monoclonal antibodies directed against a non-mite antigen. Conversely, five other antibodies completely inhibited the binding of both 3H8 G8 and 5H8 C12 to Antigen P₁ but did not inhibit the binding of 10B9 F6. These results suggest that the two groups of monoclonal antibodies

TABLE II
Inhibition of the binding of ¹²⁵I antigen P₁ to three monoclonal antibodies^a

Ascites Tested	10B9 F6		3H8 G8		5H8 C12	
	10 ⁻²	10 ⁻⁴	10 ⁻²	10 ⁻⁴	10 ⁻²	10 ⁻⁴
10B9 F6	270 99%	3,468 87%	3,895 30%	4,317 20%	11,976 41%	16,564 19%
10B9 B6	222 99%	3,938 85%	3,179 42%	4,405 20%	13,545 34%	15,094 26%
10H2 C6	150 99%	1,594 94%	2,761 50%	3,680 33%	9,704 52%	14,146 31%
3H8 G8	22,263 15%	24,344 8%	219 96%	997 82%	537 97%	5,073 75%
5H8 C12	22,429 15%	25,391 4%	163 97%	968 83%	475 98%	6,107 70%
2G7 E8	20,624 22%	21,720 17%	270 95%	1,732 69%	533 97%	9,330 54%
5B1 C12	17,950 32%	20,772 21%	182 97%	1,924 65%	405 98%	7,893 61%
3A6 A12	22,183 16%	23,739 10%	194 96%	831 85%	175 99%	1,753 91%
5H8 D8	21,200 19%	24,457 7%	3,837 30%	5,150 7%	11,577 43%	16,350 20%
Controls ^b						
8G2 B9	19,378 26%	20,057 23%	4,483 20%	4,263 23%	17,774 13%	16,158 21%
10 H11 D9	18,268 31%	20,515 22%	4,873 12%	4,596 17%	15,905 22%	15,927 22%
Mouse Antiserum to Antigen P ₁ ^c	2,943 89%	8,003 70%	335 94%	911 84%	1,930 91%	3,638 82%
No inhibitor (1% BSA PBS-T) ^d	26,305 ± 1,036		5,537 ± 615		20,430 ± 980	

^a Dilutions of ascites (10⁻² or 10⁻⁴) were used to inhibit the binding of ¹²⁵I-Antigen P₁ to monoclonal antibodies 10B9 F6, 3H8 G8, or 5H8 C12, which had been noncovalently bound to microtiter plates. Values represent quantity of ¹²⁵I-Antigen P₁ bound to plate (cpm) and percent inhibition when different ascites were used to inhibit binding. Boxes outline antibodies that caused maximum inhibition of binding of Antigen P₁ to solid-phase antibody (>96% at 10⁻² dilution and >60% at 10⁻⁴ dilution).

^b See footnotes to Table I.

^c Pooled antiserum assayed at dilutions of 1/10 and 1/50.

^d Mean ± SD of eight wells incubated with 1% BSA PBS-T.

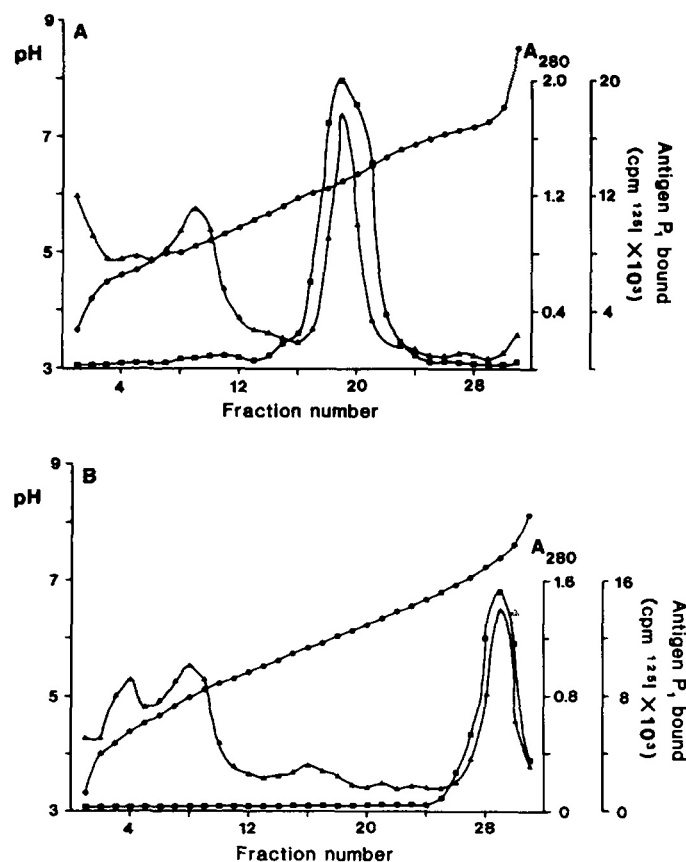


Figure 2. Purification of monoclonal antibodies by preparative IEF. Ascites from clones 10B9 F6 (A) and 5H8 C12 (B) was separated by preparative IEF, and fractions were assayed for pH (●), protein content (A₂₈₀, ▲) and IgG antibody to Antigen P₁ (■).

recognize different epitopes on Antigen P₁. One antibody, 5H8 D8, did not inhibit binding of Antigen P₁ to any of the solid-phase antibodies (Table II).

Ascites from clones 10B9 F6 (IgG1) and 5H8 C12 (IgG2a) was purified in a single-step procedure by preparative IEF (Fig. 2A and B, respectively). Each antibody focused as a single peak (isoelectric points of 6.25 [10B9 F6] and 7.4 [5H8 C12]), which contained the highest concentration of protein and showed maximum binding of Antigen P₁. These purified antibodies were radiolabeled with ¹²⁵I. Binding of both ¹²⁵I-labeled antibodies to Antigen P₁ could be fully inhibited by homologous, but not by heterologous cold antibody in cross-inhibition experiments (Fig. 3A and B). Two other monoclonal antibodies, 10H2 C6 and 3A6 A12, showed parallel inhibition curves to 10B9 F6 and 5H8 C12, respectively (Fig. 3). These results confirm that antibodies 10B9 F6 and 5H8 C12 recognized distinct epitopes on Antigen P₁. When other monoclonal antibodies were tested (Table III), they showed the same specificities as were seen in the inhibition assay by using solid-phase antibody. We considered whether the inhibition results could be explained by differences in affinity of the antibodies alone. However, the difference in inhibition of ¹²⁵I 10B9 F6 by cold 10B9 F6 and 5H8 C12 would require that 5H8 C12 had an affinity >1,000-fold less than 10B9 F6. In the reverse experiment, the opposite relationship would be required. In fact, our Scatchard analysis showed the affinity of 10B9 F6 for Antigen P₁ was fivefold greater than that of 5H8 C12. This difference could not explain the results. Thus, one group of antibodies clearly inhibited the binding of ¹²⁵I 10B9 F6 to Antigen P₁, whereas the other group only inhibited the binding of ¹²⁵I 5H8 C12.

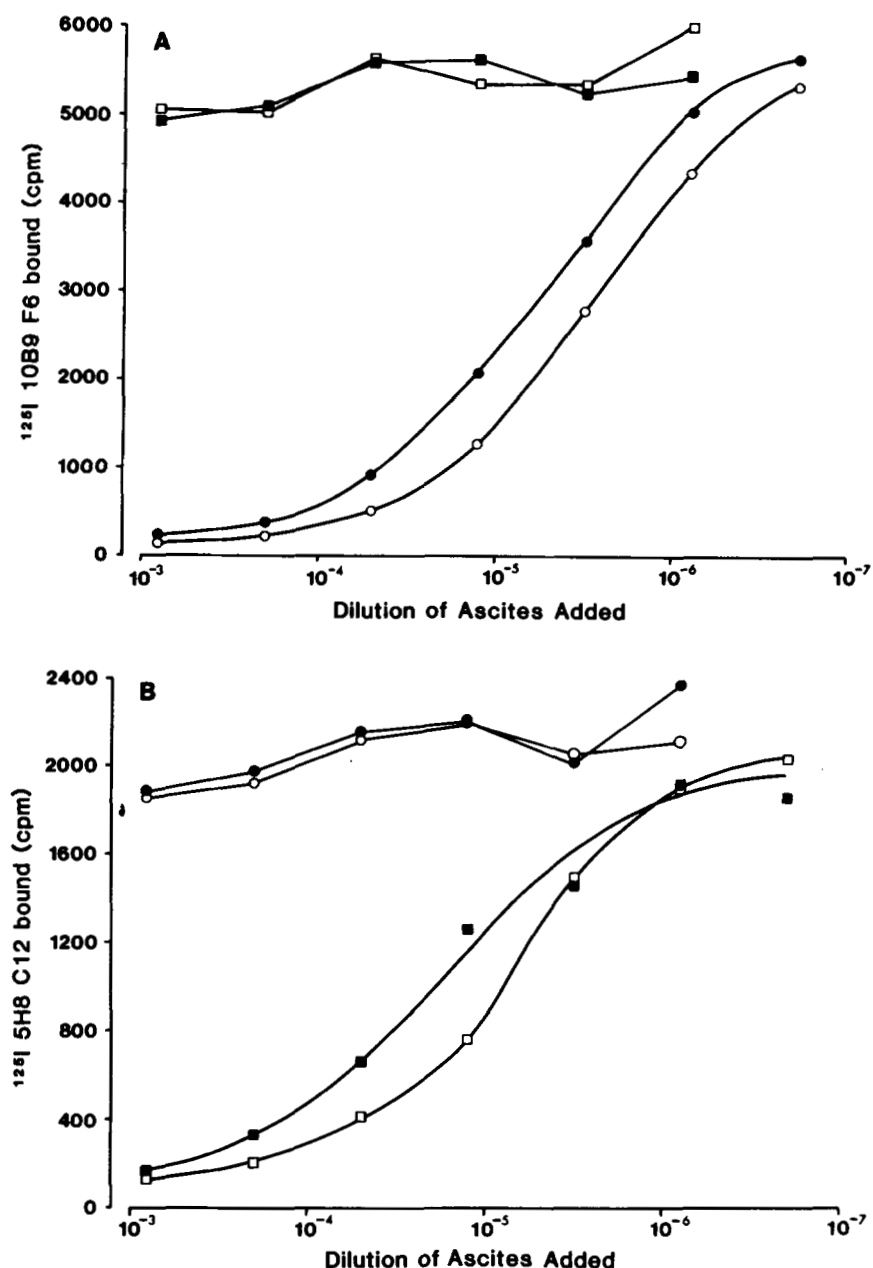


Figure 3. Inhibition of the binding of ¹²⁵I monoclonal antibodies to solid-phase Antigen P₁. Serial fourfold dilutions of ascites from clones 10B9 F6 (●), 10H2 C6 (○), 5H8 C12 (■), and 3A6 A12 (□) were used to inhibit binding of ¹²⁵I 10B9 F6 (A) or ¹²⁵I 5H8 C12 (B) to microtiter plates coated with Antigen P₁. By using control anti-*T. cruzi* ascites to inhibit, the mean quantity of ¹²⁵I bound to the solid phase was 5622 cpm for 10B9 F6 and 2492 cpm for 5H8 C12. Uninhibited binding, determined by incubating eight wells with 1% BSA PBS-T, was 6842 cpm (A) and 2663 cpm (B).

Analysis of antibodies in either supernatant or ascites from 19 clones showed that 16 recognized the same epitope as 5H8 C12 and that two recognized the same epitope as 10B9 F6. With one exception (5H8 D8), sub-clones of the same hybrid recognized the same epitope. Antibody 5H8 D8, an IgG1 sub-clone of the 5H8 hybrid, did not recognize the same epitope as 5H8 C12, which was isotype IgG2a (Table III). Analysis of supernatants from 20 parental hybrids secreting antibodies to Antigen P₁ showed a similar prevalence of antibodies binding to each epitope: 13 gave >70% inhibition against 5H8 C12, one gave >70% inhibition against 10B9 F6, four gave partial (40 to 65%) inhibition against either one or both antibodies, and two did not inhibit either antibody. Overall, 29 of the 39 clones/hybrids tested (74%) produced antibodies with the same specificity as 5H8 C12, and three out of 39 (8%) produced antibodies with the same specificity as 10B9 F6.

Inhibition of monoclonal antibody binding to Antigen

P₁ by using serum from mite-allergic patients. Initially, four monoclonal antibodies—10B9 F6, 10H2 C6, 5H8 C12, and 3H8 G8—were tested against sera from six mite-allergic patients to see whether they would inhibit the binding of human IgE antibody to Antigen P₁ in the RAST, and none of the four antibodies caused >10% inhibition. We then carried out experiments to see whether human sera would inhibit the binding of 10B9 F6 and/or 5H8 C12 to Antigen P₁. One serum (G.B.) showed >85% inhibition and parallel inhibition curves against both monoclonal antibodies (Fig. 4A). In all, 14 sera were tested, and 12 showed >20% specific inhibition of the binding of either 10B9 F6 or 5H8 C12 or both (Fig. 4B).

DISCUSSION

Immunization of mice with Antigen P₁ in CFA readily gives rise to antibody responses that can be exploited for the production of monoclonal antibodies. Our results

TABLE III
 Inhibition of the binding of ¹²⁵I-labeled monoclonal antibody to solid-phase Antigen P₁^a

Ascites Tested	10B9 F6			5H8 C12		
	10 ⁻²	10 ⁻⁴	Dilution for 50% Inhibition	10 ⁻²	10 ⁻⁴	Dilution for 50% Inhibition
10B9 F6	81 98%	478 91%	>10 ⁻⁶	1,314 37%	2,228 0%	<10 ⁻²
10B9 B6	80 99%	400 92%	>10 ⁻⁶	1,422 32%	1,728 17%	<10 ⁻²
10H2 C6	84 98%	259 95%	>10 ⁻⁶	1,516 27%	1,642 21%	<10 ⁻²
3H8 G8	3,936 24%	4,451 12%	<10 ⁻²	72 97%	475 77%	>10 ⁻⁶
5H8 C12	3,714 28%	4,577 11%	<10 ⁻²	65 97%	394 81%	>5 × 10 ⁻⁴
2G7 E8	3,907 24%	4,438 14%	<10 ⁻²	79 96%	496 76%	>10 ⁻⁶
5B1 C12	3,769 27%	4,659 10%	<10 ⁻²	80 96%	364 83%	>10 ⁻⁶
3A6 A12	3,729 28%	4,176 19%	<10 ⁻²	70 97%	215 87%	>10 ⁻⁶
5H8 D8	2,928 43%	4,605 11%	<10 ⁻²	1,541 26%	1,960 6%	<10 ⁻²
Anti- <i>T. cruzi</i> antibodies ^b	4,364 ± 373 16 ± 0.9%	ND ^c	—	1,657 ± 189 20 ± 0.9%	ND	—
Mouse anti-serum to Antigen P ₁ ^c	1,725 67%	3,613 30%	4 × 10 ⁻²	766 63%	1,208 42%	3 × 10 ⁻²
No inhibitor (1% BSA PBS-T) ^d	5,170 ± 221			2,083 ± 190		

^a Dilutions of ascites (10⁻² and 10⁻⁴) were used to inhibit binding of ¹²⁵I-labeled 10B9 F6 or 5H8 C12 to microtiter plates coated with Antigen P₁-enriched mite extract (*D. pteronyssinus* 50% SAS cut). Quantity of ¹²⁵I-labeled antibody bound (cpm) and percent inhibition are shown. Boxes outline groups of monoclonal antibodies that recognize same epitope as either 10B9 F6 or 5H8 C12.

^b Mean ± SD for antibodies 8G2 B9 and 10H11 D9.

^c Tested at dilutions of 10⁻² and 10⁻³.

^d Mean SD of eight wells incubated with 1% BSA PBS-T.

^e ND = not determined.

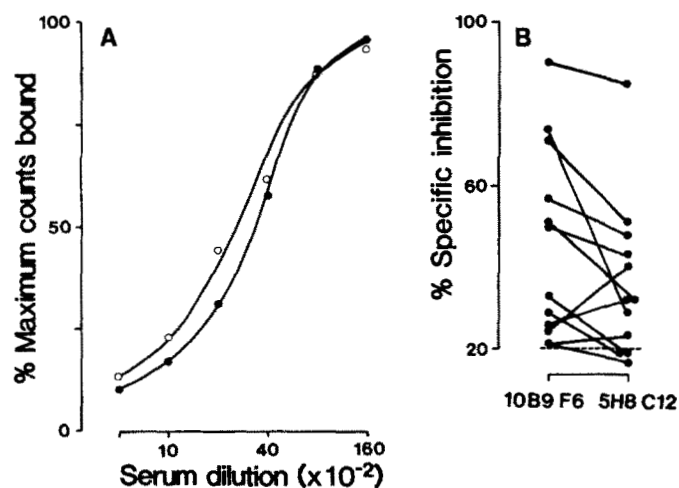


Figure 4. Inhibition of the binding of ¹²⁵I 10B9 F6 and ¹²⁵I 5H8 C12 to Antigen P₁ by serum from mite-allergic patients. Doubling dilutions of serum from patient G.B. were used to inhibit the binding of 10B9 F6 (●) or 5H8 C12 (○) to solid-phase Antigen P₁ (A). This patient had high levels of serum IgG and IgE antibody to Antigen P₁ (12,500 U/ml and 400 U/ml, respectively). Sera from 12 patients were tested at 1/5 dilution for inhibition of each ¹²⁵I-labeled antibody (B). (Top line in B = serum from G.B.) Values for percent specific inhibition were obtained after subtracting the mean percent inhibition obtained by using four sera from nonallergic controls (see Materials and Methods), i.e., 30% and 32% of the uninhibited binding of 10B9 F6 and 5H8 C12, respectively. In these experiments, 15,000 cpm 10B9 F6 and 5700 cpm 5H8 C12 were bound in the absence of inhibitor (1% BSA PBS-T controls).

show that the majority of monoclonal antibodies produced from this fusion were Antigen P₁ specific and isotype IgG1. Moreover, these antibodies were also *D. pteronyssinus* specific and did not react with the analogous allergen from *D. farinae*. This finding was surprising because rabbit antisera show cross-reactivity be-

tween both these mite allergens. Allergic patients also show immediate hypersensitivity to allergens derived from both mite species (26, 27). Antigen-binding assays with sera from the 10 mice that had been immunized with Antigen P₁, as well as the pooled mouse antiserum to Antigen P₁, showed that these sera contained much lower levels of IgG antibody to *D. farinae* Fr. I (data not shown). These preliminary studies, therefore, raise the possibility that mouse IgG antibodies produced after immunization with antigen P₁ in CFA are selectively directed against *D. pteronyssinus*-specific antigenic determinants.

Inhibition experiments with the monoclonal antibodies in two different solid-phase radioimmune binding assays showed that these antibodies recognized two epitopes on Antigen P₁. There was some nonspecific inhibition in both assays, particularly in the assay involving inhibition of ¹²⁵I Antigen P₁ binding to solid-phase antibody. However, given that several antibodies bound 40 to 50% ¹²⁵I Antigen P₁ in the fluid phase, they might well be expected to reduce the small quantity of radiolabel (10 ng) available for binding to a solid-phase antibody. Concordant results were obtained in both assays, and the cross-inhibition curves clearly distinguished two epitopes based on the binding of either 10B9 F6 or 5H8 C12 monoclonal antibody. A high proportion (74%) of antibodies produced in this fusion were directed against the epitope recognized by 5H8 C12, suggesting that this could be an "immunodominant" determinant. From comparisons of the dilutions of ascites required for 50% inhibition of binding to each epitope, it seems clear that these epitopes are non-overlapping antigenic sites on Antigen P₁. It is also possible that there are other *D. pteronyssinus*-specific determinants on Antigen P₁. One monoclonal antibody (5H8

D8) and antibodies from two parental lines failed to inhibit the binding of either 10B9 F6 or 5H8 C12, suggesting that they might be directed against other epitopes.

Allergen-specific monoclonal antibodies have considerable potential for use in several areas of research on immediate hypersensitivity. These include i) identification and quantitation of allergens in commercial extracts for standardization purposes (21, 22, 28), ii) analysis of allergen structure, iii) analysis of antigenic/allergenic determinants that stimulate both IgG and IgE antibody responses and T cells in mite-allergic patients. In addition, monoclonal antibodies to allergens known to be present in house dust (e.g., Antigen P₁, Cat I, and Alt-I) would be of particular value for measuring the quantities of these allergens in dust samples from allergic patients' houses. Recently, an International Standard *D. pteronyssinus* extract of known Antigen P₁ content was established (NIBSC 82/518) (29). This standard will serve as a reference to enable different laboratories to compare the Antigen P₁ content of mite extracts and house dust extracts. Our results leave little doubt that the monoclonal antibodies described here could be used as primary reagents for the identification of Antigen P₁ in allergen extracts containing *D. pteronyssinus*. These antibodies could replace conventional antisera in quantitative assays, e.g. radioimmunoassay. However, they would not be suitable for assays of the allergen content of *D. farinae* extracts. This would best be achieved by the production of monoclonal antibodies to common determinants on both Antigen P₁ and the cross-reacting *D. farinae* allergen.

The availability of monoclonal antibodies to Antigen P₁ is a first step toward defining the antigenic structure of this glycoprotein. Structural studies with globular proteins, e.g., myoglobin, lysozyme, and albumin (reviewed in Reference 30), clearly show that assessments of antigenicity will also depend on primary amino acid sequence and three dimensional protein conformation. There have been few such studies in relation to allergens. However, ragweed allergen Ra₅ has been sequenced (31), and there have been preliminary studies on the binding of isoallergens (32) and chemically modified Ra₃ (33) by human IgE antibodies. In the present studies we found that, whereas human sera inhibited binding of monoclonal antibodies to Antigen P₁, individual monoclonal antibodies had very little effect on human IgE binding in RAST. This suggests that human antibodies are directed against multiple antigenic determinants such that blocking one epitope with a single monoclonal antibody would not cause significant inhibition. With the production of more monoclonal antibodies to the same allergen, it should be possible to identify the determinants recognized both by human IgG and IgE antibodies and also by human T cells (12) by using inhibition experiments. Studies on T cells would attempt to answer whether T cells from patients with different diseases associated with immediate hypersensitivity are directed against the same or different epitopes (11, 12). By using a panel of monoclonal antibodies, it would be possible to map the antigenic structure of Antigen P₁ as has been done for globular proteins (30) and viral glycoproteins (34). In addition, it should be possible to answer whether cross-linking of the surface of a mast cell with Antigen P₁ requires IgE antibodies to two different epitopes. Finally, we would like to study whether

patients of different ages, with different diseases or with different total levels of serum IgE, have IgE antibodies directed against different epitopes or a different number of epitopes.

Acknowledgments. We thank Bencard Ltd. and Hollister-Stier Ltd. for generous supplies of *D. pteronyssinus* and *D. farinae* culture; Dr. R. Taylor, Department of Biochemistry, University of Virginia, for advice on the measurement of affinity; Dr. T. Smith, Emory University, Atlanta, for providing two of the sera from mite allergic patients; Madeleine Watkins for drawing the figures; and Lee Ann Fargo for secretarial assistance.

REFERENCES

1. Voorhorst, R., F. Th. M. Spijksma, H. Verekamp, M. J. Leupen, and A. W. Lyklema. 1967. The house dust mite (*Dermatophagoides pteronyssinus*) and the allergens it produces. Identity with the house dust allergen. *J. Allergy* 39:325.
2. Smith, J. M., M. E. Disney, J. P. Williams, and Z. A. Goels. 1969. Clinical significance of skin reactions to mite extracts in children with asthma. *Br. Med. J.* 2:723.
3. Chapman, M. D., and T. A. E. Platts-Mills. 1980. Purification and characterization of the major allergen from *Dermatophagoides pteronyssinus*—antigen P₁. *J. Immunol.* 125:587.
4. Tovey, E. R., M. D. Chapman, and T. A. E. Platts-Mills. 1981. Mite faeces are a major source of house dust allergens. *Nature* 289:592.
5. Tovey, E. R., M. D. Chapman, C. W. Wells, and T. A. E. Platts-Mills. 1981. The distribution of dust mite allergen in the houses of patients with asthma. *Am. Rev. Respir. Dis.* 124:630.
6. Lind, P., B. Weeke, and H. Löwenstein. A reference allergen preparation of the house dust mite *D. pteronyssinus* produced from whole mite culture. A part of the DAS 76 study. *Allergy* 39:259.
7. Stewart, G. A., and K. J. Turner. 1980. Physicochemical and immunological characterization of the allergens from the mite *Dermatophagoides pteronyssinus*. *Aust. J. Exp. Biol. Med. Sci.* 58:259.
8. Stewart, G. A. 1982. The isolation and characterization of the allergen Dpt 12 from *Dermatophagoides pteronyssinus* by chromatofocusing. *Int. Arch. Allergy Appl. Immunol.* 69:224.
9. Chapman, M. D., E. R. Tovey, S. R. Wilkins, and T. A. E. Platts-Mills. 1983. The allergens responsible for house dust mite allergy. In *Proceedings of the XI International Congress of Allergy and Clinical Immunology*, London, 1982. Macmillan Press, London. P. 531.
10. Chapman, M. D., and T. A. E. Platts-Mills. 1978. Measurement of IgG, IgA, and IgE antibodies to *Dermatophagoides pteronyssinus* by antigen-binding assay, using a partially purified fraction of mite extract (F_{P1}). *Clin. Exp. Immunol.* 34:126.
11. Chapman, M. D., S. R. Rowntree, M. C. di Prisco de Fuenmayor, E. B. Mitchell, and T. A. E. Platts-Mills. 1983. Quantitative assessments of IgG and IgE antibodies to inhalant allergens in patients with atopic dermatitis. *J. Allergy Clin. Immunol.* 72:27.
12. Rawle, F. R., E. B. Mitchell, and T. A. E. Platts-Mills. T cell responses to the major allergen from the house dust mite *Dermatophagoides pteronyssinus*. Antigen P₁: comparison of patients with asthma, atopic dermatitis, and perennial rhinitis. *J. Immunol.* 133:195.
13. Mitchell, E. B., J. Crow, M. D. Chapman, S. S. Jouhal, F. M. Pope, and T. A. E. Platts-Mills. 1982. Basophils in allergen-induced patch test sites in atopic dermatitis. *Lancet* i:127.
14. Mitchell, E. B., J. Crow, S. Rowntree, A. D. B. Webster, and T. A. E. Platts-Mills. Cutaneous basophil hypersensitivity to inhalant allergens: local transfer of basophil accumulation with immune serum but not IgE antibody. *J. Invest. Dermatol.* In press.
15. Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256:495.
16. Smart, I. R., J. Heddle, H. Zola, and J. Bradley. 1983. Development of monoclonal mouse antibodies specific for allergenic components in rye grass (*Lolium perenne*) pollen. *Int. Arch. Allergy Appl. Immunol.* 72:243.
17. Kahn, C., and D. G. Marsh. 1983. Analysis of grass group I allergens using monoclonal antibodies. *J. Allergy Clin. Immunol.* 71:95. (Abstr.)
18. Krilis, S., B. A. Baldo, R. L. Raison, R. E. Callard, and A. Basten. 1983. Standardization of antigen E in ragweed pollen extracts using a monoclonal antibody based enzyme immunoassay. *J. Allergy Clin. Immunol.* 71:261.
19. Theobald, K., A. Bohn, H. Thiel, B. Rasche, W. T. Ulmer, and W. König. 1983. Production of monoclonal antibodies against wheat flour components. *Int. Arch. Allergy Appl. Immunol.* 72:84.
20. Agarwal, M. K., R. M. Helm, K. Hisamatsu, G. J. Gleich, and J. W. Yunginger. 1983. Monoclonal antibody to ALT-I and its use in affin-

- ity chromatography. *J. Allergy Clin. Immunol.* 71:160 (Abstr.)
21. Krilis, S., B. A. Baldo, and A. Basten. 1981. Standardization of allergens: lectins and monoclonal antibodies as primary standards for house dust mite allergens. *Int. Arch. Allergy Appl. Immunol. (Suppl. 1)* 66:252.
 22. Lind, P., and Aalberse, R. Crossed immunoelectrophoretic (CIE) studies of monoclonal antibodies to allergens from cat and house dust mite. *Proceedings of the XV Annual Meeting of the Scandinavian Society for Immunology*. In Press.
 23. Chapman, M. D., E. R. Tovey, and T. A. E. Platts-Mills. 1982. Physical properties and origin of house dust mite allergens. *Folia Allergol. Immunol. Clin.* 29:25.
 24. Shulman, M., C. D. Wilde, and G. Kohler. 1978. A better cell line for making hybridomas secreting specific antibodies. *Nature* 276:269.
 25. Chapman, M. D., D. Snary, and M. A. Miles. 1984. Quantitative differences in the expression of a cell surface glycoprotein (GP72) in *Trypanosoma cruzi* zymodemes. *J. Immunol.* 132:3149.
 26. Voorhorst, R., and F. Th. M. Spijkema. 1969. Comparison of skin reactions to extracts of house dust, mites, and human skin scales. *Acta Allergologica* 24:124.
 27. Pepys, J., M. Chan, and M. E. Hargreave. 1968. Mites and house dust allergy. *Lancet* i:1270.
 28. Baldo, B. A. 1983. Standardization of allergens. Examination of existing procedures and the likely impact of new techniques on the quality control of extracts. *Allergy* 38:535.
 29. Ford, A. W., F. C. Rawle, P. Lind, F. Th. M., Spijkema, H. Löwenstein, and T. A. E. Platts-Mills. 1984. Standardization of *Dermatophagoides pteronyssinus*: assessment of potency and allergen content in ten coded extracts. *Int. Arch. Allergy Appl. Immunol.* In Press.
 30. Benjamin, D. C., J. A. Berzofsky, I. J. East, F. R. N. Gurd, C. Hannum, S. J. Leach, E. Margoliash, J. G. Michael, A. Miller, E. M. Prager, M. Riechlin, E. E. Sercarz, S. J. Smith-Gill, P. E. Todd, and A. C. Wilson. 1984. The antigenic structure of proteins: a reappraisal. *Annu. Rev. Immunol.* 2:67.
 31. Mole, L. E., L. Goodfriend, C. B. Lapkoff, J. M. Kehoe, and J. C. Capra. 1975. The amino acid sequence of ragweed pollen allergen Ra5. *Biochemistry* 14:1216.
 32. Roebber, M., D. G. Klapper, and D. G. Marsh. 1983. Structure and function relationship of the ragweed pollen isoallergens, Ra5A and Ra5B. *J. Allergy Clin. Immunol.* 71:162. (Abstr.)
 33. Chang, J. Y., G. J. Gleich, and M. Z. Atassi. 1981. The role of the thiol group and the disulphide bond in the conformation and the IgE and IgG binding activities of ragweed allergen Ra3. *Mol. Immunol.* 18:991.
 34. Colman, P. M., J. N. Varchese, and W. G. Laver. 1983. Structure of the catalytic and antigenic sites in influenza virus neuraminidase. *Nature* 303:41.