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Cloned human T lymphocytes reactive with *Dermatophagoides farinae* (house dust mite): a comparison of T- and B-cell antigen recognition

R. E. O'HEHIR, D. B. YOUNG,* A. B. KAY & J. R. LAMB* *Department of Allergy and Clinical Immunology, Cardiothoracic Institute, Brompton Hospital and *MRC Tuberculosis and Related Infections Unit, Hammersmith Hospital, London*

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SUMMARY

In this report, T-cell and B-cell recognition of the house dust mite *Dermatophagoides farinae* (*D. far.*) is compared. Nitrocellulose immunoblots of polyacrylamide gel electrophoresis (SDS–PAGE)-fractionated *D. far.* were added to proliferation assays to map the antigen specificity of cloned human helper T cells and a long-term line induced with *D. far.* T-cell recognition was of a polypeptide of molecular weight 9000–13,000, that migrates with the serologically defined allergen *Der f* II (12,500 MW). Since the cloned T cells, unlike the polyclonal response, failed to respond to *Dermatophagoides pteronyssinus* (*D. pter.*), this suggests that they recognize a species-specific epitope. In contrast, analysis of the B-cell response using Western blotting demonstrated that, in addition to *Der f* II, antibodies reactive with the major allergens *Der f* I (26,000 MW) and *Der f* III (29,000 MW) were present in the serum. Similar specificities were seen in the antibody response to *D. pter.*, and while it has been reported that the B-cell response to *D. far.* and *D. pter.* are predominantly cross-reactive, our observations suggest that species-specific CD4-positive T cells are present in the overall cellular response to *D. far.*

INTRODUCTION

The T-cell dependency and reaginic activity of IgE in clinical allergy is well documented and, unlike other classes of immunoglobulin, rather than being protective is responsible for allergic sensitivity in atopic individuals (Ishizaka, 1984). Early studies (Orange, Austen & Austen, 1971) demonstated that allergen–IgE antibody reactions in the human lung induce chemical mediators that cause the symptoms of respiratory allergy.

In contrast, the antigen specificity and functional role of T lymphocytes in the induction and regulation of allergic respiratory diseases remain ill defined, with current research restricted largely to phenotypic analysis (Metzger *et al.*, 1987). Recognition of allergens such as ragweed (Meuer *et al.*, 1983), pollen (Brostoff, Greaves & Roitt, 1969) and *Dermatophagoides* (Rawle, Mitchell & Platts-Mills, 1984; Lanzavecchia *et al.*, 1983) by the T cells of atopic individuals has been reported. Rawle *et al.* (1984) observed that helper T cells from atopic but not control individuals responded to the purified major allergen pI from the house dust mite *D. pter.* Lanzavecchia *et al.* (1983) isolated HLA-DR-restricted T-cell lines and clones reactive with the aeroallergen *D. pter.* They subsequently used alloreactive T-helper clones for the *in vitro* stimulation of IgE production (Lanzavecchia & Parodi, 1984).

Investigation of the antigenic structures of the house dust mites *D. pter.* and *D. far.* suggest that *Der p* I and *Der f* I, both of molecular weight 24,000, are the major allergens (Chapman & Platts-Mills, 1980; Heymann, Chapman & Platts-Mills, 1986). Others have reported major allergens of slightly different molecular weights, but it has been accepted generally that the most allergenic fractions were distributed in the range of 20,000–30,000 (Dandeu *et al.*, 1982; Stewart & Turner, 1980). Additionally, *Der f* II, a major allergen (12,500 MW), and other minor allergens have also been described (Heymann *et al.*, 1987). However, the identification of these antigens was based on monoclonal antibodies, and since T and B cells may recognize different components of *D. far.*, this should be supplemented by a direct approach for the analysis of T-cell antigen recognition.

In this study, in order to investigate the role of cell-mediated immunity in the allergic response to house dust mite, T-cell lines and clones reactive with *D. far.* were isolated. Using the SDS–PAGE immunoblot assay system for T-cell antigen recognition (Young & Lamb, 1986) the fine specificity could be mapped to determinants present in the molecular range of 9000–13,000 and

Abbreviations: APC, antigen-presenting cell; *D. far.*, *Dermatophagoides farinae*; *D. pter.*, *Dermatophagoides pteronyssinus*; [^{3}H]TdR, tritiated methyl thymidine; PBMC, peripheral blood mononuclear leucocytes; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis.

Correspondence: Dr R. E. O'Hehir, Dept. of Allergy and Clinical Immunology, Cardiothoracic Institute, Brompton Hospital, Fulham Road, London SW3 6HP, U.K.

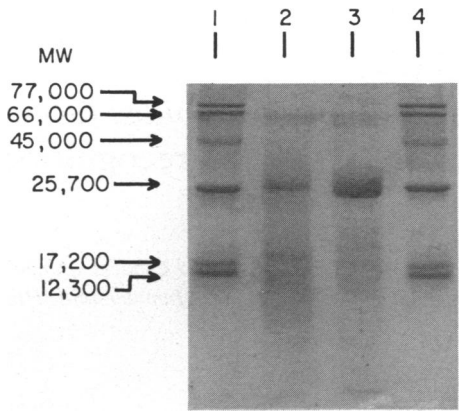

Figure 1. SDS–polyacrylamide gel of *D. far.* and *D. pter.* extracts. *D. far.* extract (10^5 BU protein per lane) was prepared and run on SDS–polyacrylamide gel, and proteins were blotted onto nitrocellulose. A representative gel stained with Coomassie blue is shown with molecular weight markers. *D. pter.* extract was run in parallel (*D. pter.*, Lane 3; *D. far.*, Lane 2; molecular weight markers, Lanes 1 and 4).

was specific for *D. far.*, showing no recognition of the closely related genus *D. pter.* In contrast, the IgE antibody response to *Der f* II was cross-reactive.

MATERIALS AND METHODS

Antigens

Lyophilized extracts of *D. far.*, Timothy grass, five-grass mix and Parietaria were kindly provided by Pharmacia (Uppsala, Sweden). *D. pter.* was the generous gift of Bencard (Brentford, Middlesex).

Antibodies

Monoclonal antibodies reactive with T-cell surface antigen, Leu 2a, Leu 3a, Leu 4, HLA-DR and IL-2 receptor were purchased from Becton-Dickinson, Sunnyvale, CA.

Preparation of immunoblots

SDS–PAGE immunoblots were prepared as described previously (Young & Lamb, 1986). Briefly, antigen was boiled in the presence of SDS and 2-mercaptoethanol and separated by SDS–polyacrylamide gel electrophoresis in a 'mini-gel' apparatus (Hoeffer Scientific, San Francisco, CA) using the Laemmli system (Laemmli, 1970) with an acrylamide concentration of 15% (w/v). Samples of *D. far.* extract for electrophoresis contained 10^5 biological units (BU) of protein per lane. Proteins were transferred from gels to nitrocellulose by electroblotting as described by Towbin, Staehelin & Gordon (1979). Blots were reversibly stained by dipping for a few seconds in Amido black (0·1% in 0·5% acetic acid) and washed with distilled water to permit accurate localization of protein lanes (Fig. 1). Blots were further washed with 0·1% Triton X-100 in phosphate-buffered saline (PBS) and then with PBS alone prior to use. Each nitrocellulose blot (0·5 × 4 cm) was divided into 20 equal sections. These sections were then solubilized using the modification of Abou-Zeid *et al.* (1987). Dimethylsulphoxide (1 ml) was added to each section for 1 hr at room temperature followed by an equal volume of bicarbonate/carbonate buffer, pH 9·6, to allow reprecipitation. The preparations were then precipitated in a microfuge at 10,000 r.p.m. and washed four times with RPMI-1640 medium (Flow, Irvine, Ayrshire) and resuspended to a final volume of 0·5 ml, of which 20 μl were added to each well in proliferation assays. Molecular weights were determined from a standard curve obtained using molecular weight markers (Sigma, Poole, Dorset).

Preparation of lymphocytes

Peripheral blood mononuclear leucocytes (PBMC) obtained from a healthy adult with perennial rhinitis and a positive skin prick test to the house dust mite *D. far.* were isolated by centrifugation on a discontinuous gradient of Ficoll–Paque (Pharmacia).

Isolation of antigen-reactive T-lymphocyte lines and clones

Antigen-reactive T-cell line and clones were isolated as described previously (Lamb *et al.*, 1982). Briefly, PBMC ($2·5 \times 10^5$/ml) were cultured with *D. far.* antigen (10^3 BU/ml) for 7 days in RPMI-1640 supplemented with 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin and 5% screened AB$^-$ serum. Lymphoblasts enriched on Ficoll–Paque were established as a long-term line in the presence of irradiated (2500 rads) autologous PBMC, *D. far.* and interleukin-2 (IL-2; Lymphocult T, Biotest Folex, Frankfurt, FRG) or cloned by limiting dilution from the line. For cloning, lymphoblasts ($33\frac{1}{3}$ cells/ml) were plated in Microtest II trays together with irradiated autologous PBMC (5×10^5/ml), *D. far.* (10^3 BU/ml) and IL-2 (Lymphocult T 5% v/v supplemented with recombinant IL-2, 5 U/ml, the generous gift of Boehringer, Mannheim, FRG). After 7 days, growing clones were transferred to flat-bottomed 96-well microtitre trays and subsequently to 24-well trays. At each transfer the clones received fresh IL-2 and irradiated autologous PBMC together with specific antigen (*D. far.*; 10^3 BU/ml). The clones were maintained with further IL-2 every 3–4 days, and irradiated autologous PBMC and *D. far.* were added every 7 days. Before use in proliferation assays, the clones were rested for 6–8 days after the last addition of filler cells and antigen.

Proliferation assays

T cells of the long-term line (DX2) and clone (DD11; 5×10^4/ml) were cultured with soluble or insoluble antigen in the presence of autologous irradiated PBMC (5×10^5/ml) in 96-well flat-bottomed microtitre plates, as described previously (Young & Lamb, 1986). After a 72 hr incubation, the cultures were pulsed with 0·66 μCi of tritiated methyl thymidine ([^{3}H]TdR; Radiochemicals Inc., Amersham, Bucks, U.K.) for 8–16 hr, then harvested onto glass-fibre filters.

Proliferation as correlated with [^{3}H]TdR incorporation was measured by liquid scintillation spectroscopy. The results are expressed as the mean counts per minute (c.p.m. ± SEM). The duration of unfractionated PBMC cultures (10^5/well) was 7 days.

Autoradiography from immunoblots of D. far. *and* D. pter.

Nitrocellulose-transferred SDS–PAGE immunoblots of *D. far.* and *D. pter.* were prepared as described. Western blotting and autoradiography were performed using the modification of Tee *et al.* (1987). Briefly, non-specific binding was blocked by immersing the nitrocellulose sheets in 2% human serum albumin in Tris buffer (pH 7·5) for 1 hr at 39°. After washing, a range of concentrations of autologous serum (undiluted, 1:2, 1:5) was allowed to react over the nitrocellulose sheets for 16 hr. After

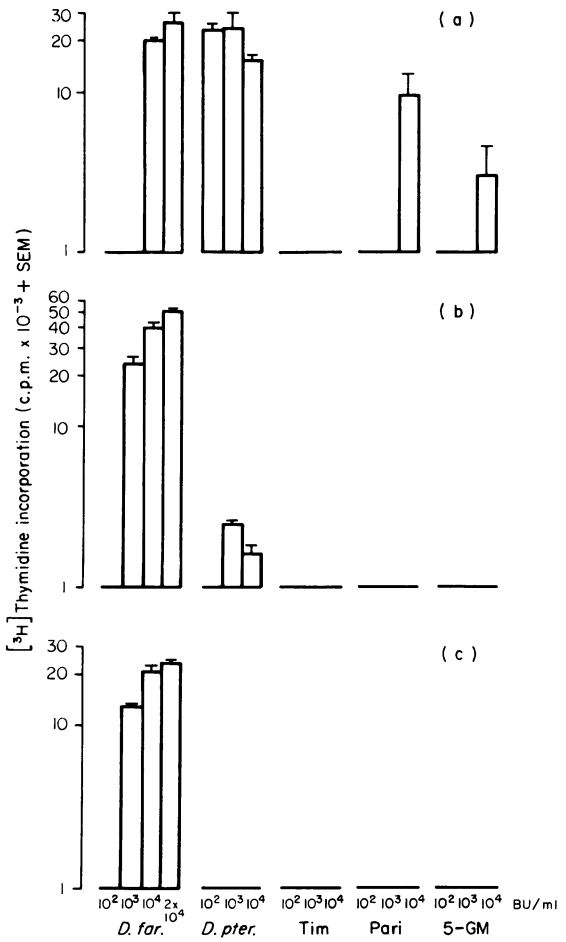

Figure 2. T-cell proliferative responses to a panel of soluble aeroallergens. (a) PBMC (10^5/well) were cultured with soluble *D. far.*, *D. pter.*, Timothy grass, Parietaria and five-grass mix. Proliferation was determined by [^{3}H]TdR incorporation in a 7-day assay. The results are expressed as counts per minute (c.p.m.) ± SEM of triplicate cultures. Background response of PBMC in the absence of antigen was 349 ± 48 c.p.m. (b) T cells of line (DX2) and (c) clone (DD11) (10^4 well) were stimulated with the same aeroallergens together with autologous irradiated PBMC (5×10^4/well). [^{3}H]TdR incorporation was determined at 72 hr. Background responses of the DX2 and DD11 to APC in the absence of antigen were 87 ± 19 and 95 ± 30 c.p.m., respectively.

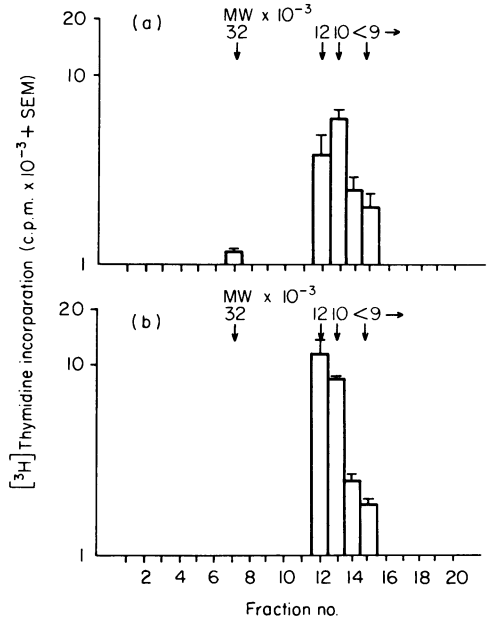

Figure 3. Differential pattern of reactivity of the T-cell line (a) and clone (b) to SDS–PAGE antigens solubilized from immunoblots of *D. far.* T cells of the *D. far.*-specific line (DX2) and the clone (DD11) (10^5/ml) were cultured with SDS–PAGE-separated immunoblots (20 fractions) after solubilization with DMSO, together with irradiated PBMC (5×10^5/ml) and assayed as described in the legend to Fig. 2b and c. Background response of DX2 and DD11 to APC in the absence of antigen was 973 ± 20 and 66 ± 16 c.p.m., respectively.

washing, this was followed by a further 16-hour incubation with the tracer ^{125}I anti-IgE (Pharmacia) (100 μl/approximately 10^4 c.p.m.). The washed, dried sheets were exposed to X-OMAT L Kodak film with Du Pont Quanta III intensifying screens at −70° for 48 hr, and then developed. Negative controls using serum (1:2 and 1:5) from a non-atopic individual were processed in parallel.

Cytofluorimetric analysis

T cells were incubated with monoclonal antibodies Leu 2a, Leu 3a, Leu 4, HLA-DR and anti-IL-2R followed by FITC-conjugated rabbit anti-mouse immunoglobulin ($F(ab)_2$; Dakopatts, High Wycombe, Bucks, U.K.). The cells were analysed for fluorescence on a FACS-analyser cytofluorimeter (Becton-Dickinson).

RESULTS

T-cell proliferation in response to soluble *D. far.* antigen

PBMC from a patient with perennial rhinitis proliferated in a dose-dependent manner when stimulated with soluble *D. far.* extract (Fig. 2a). In addition, proliferative responses were also observed to the other aeroallergens as compared to the medium control. Although no response was observed to Timothy, the biological activity of the allergen was confirmed in sensitized patients. Both the long-term T-cell line (DX2) and cloned T cells (DD11) proliferated in response to the inducing antigen *D. far.*, but failed to recognize the allergens Timothy, Parietaria and five-grass mix over a range of concentrations. DX2 gave a limited proliferative response to *D. pter.*, but DD11 showed no recognition of this allergen (Fig. 2b and c). This confirms the specificity of the T-cell line and clone for *D. far.*

Reactivity pattern of T-cell line and clone to SDS–PAGE immunoblots of *D. far.*

T lymphocytes from the line and clone in the presence of accessory cells were observed to proliferate in response to *D. far.* supplied in particulate form bound to nitrocellulose after fractionation on SDS–PAGE (Fig. 3). The long-term T-cell line (DX2) predominantly recognized antigenic determinants within the molecular weight range 9000–13,000 (Fig. 3a) which contained the only determinant recognized by the clone DD11 (Fig. 3b). In addition, the line showed minimal recognition of the protein band (24,000–29,000) that would correspond to the

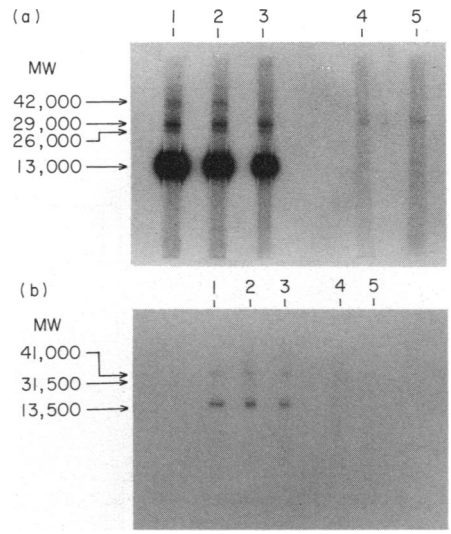

Figure 4. Autoradiographs of Western blots showing the IgE response of autologous and non-atopic allogeneic sera to *D. far.* and *D. pter.* antigens. *D. far.* (a) and *D. pter.* (b) mite body extracts were separated by SDS–PAGE and transferred to nitrocellulose. The strips were probed with sera from the mite-allergic PBMC donor (undiluted, Lane 1; 1:2, Lane 2; and 1:5, Lane 3) and from a non-allergic control (1:2, Lane 4; and 1:5, Lane 5) and autoradiographed to detect binding of IgE as described in the Materials and Methods.

Der f I and *Der f* III allergens. To control for the possibility that proliferation was a non-specific mitogenic effect, all 20 fractions were added in the presence of accessory cells to cloned T cells reactive with influenza haemagglutinin (Lamb & Green, 1983), with the result that no proliferation was observed. Neither were any of the fractions able to inhibit the response of this clone to influenza, thus eliminating the presence of non-specific inhibitors (data not shown). These results would suggest that the proteins in the molecular weight range 9000–13,000 constitute the major component of T-cell recognition for this individual.

Reactivity pattern of specific IgE to immunoblots of *D. far.*

The autoradiographs using autologous serum showed strong IgE binding at the 13,000 fraction corresponding to the major site of T-cell recognition (Fig. 4a). Fainter bands were demonstrable at 26,000, 29,000 and 42,000 MW. These first two bands correspond to the molecular weights of *Der f* I and *Der f* III, and reflect the minor recognition site of the T-cell line DX2. Similarly, the pattern of binding to *D. pter.* was predominantly against the 13,500 MW band, with additional bands at 41,000 and 31,500 (Fig. 4b).

Interestingly, the major protein band on this gel was consistent with *Der p* I (Fig. 1). None of these antigens was recognized by the control sera.

Phenotypic analysis of cloned T lymphocytes

Since the T cells showed identical specificity to the anti-*D. far.* IgE response (Fig. 4a), it was of interest to determine if those T cells were of the helper phenotype. Both the long-term T-cell line DX2 and clone DD11 expressed CD3 and MHC class II determinants characteristic of activated mature peripheral T cells. The clone DD11 was CD4-positive and therefore of the helper/inducer population. The line was also predominantly CD4-positive, although there was a minor component of CD8 (suppressor/cytotoxic T subsets)-positive T cells. The reduced expression of IL-2R in clone DD11 as compared to the line may reflect the state of activation (IL-2R down-regulation) since the cells were rested for 6–8 days after the last addition of filler cells and antigen before phenotypic analysis (Fig. 5).

DISCUSSION

The T-cell dependency of antibody production in allergic disease is well established (Ishizaka, 1984). However, the specificity and regulatory role of T cells in the immune response to house dust mite remain to be explored.

In this study the antigen specificity of both the polyclonal and clonal response of a patient with perennial rhinitis attributable to house dust mite allergy has been analysed. At the polyclonal level, both *D. far.* and the closely related house dust mite *D. pter.* were able to induce marked T-cell proliferation. Therefore, in order to determine whether or not T-cell recognition of *D. far.* was limited to cross-reactive determinants also present on *D. pter.*, an IL-2-dependent T-cell line and clone induced with *D. far.* were isolated. Similar to the polyclonal response, the T-cell line responded to both *D. far.* and *D. pter.*, although the magnitude of the response to the latter was considerably less, suggesting that T-cell recognition was directed mainly to species-specific determinant(s). Indeed, the cloned T cells also appeared to recognize a species-specific antigen of *D. far.*

The information available on T-cell recognition of the genus *Dermatophagoides* is limited. Rawle *et al.* (1984) described polyclonal T-cell proliferation to the pI antigen of *D. pter.*; however, whether or not the responses were cross-reactive with *D. far.* was not investigated. Certainly it would appear that human IgE antibody response to *Der p* I and *Der f* I is predominantly cross-reactive (80–95%; Chapman, Heymann & Platts-Mills, 1987), and although T and B cells may recognize different determinants within a protein antigen (Berzofsky, Richman & Killon, 1979; Lamb & Green , 1983), the specificity of the T-cell response may parallel that observed for B cells. In order to determine whether the T-cell response was directed towards *Der f* I, the reputed major allergen of *D. far.*, nitrocellulose immunoblots of SDS–PAGE-separated antigen were prepared and added to T-cell proliferation assays. Using this approach the determinant(s) recognized by both the T-cell line and clone could be mapped in the molecular range of 9000–13,000. This co-migrates with *Der f* II (MW 12,500), recently proposed by Heymann *et al.* (1987) to be a major allergen of *D. far.* in addition to the 24,000 MW protein *Der f* I (Heymann *et al.*, 1986). Interestingly, the results reported would suggest that the T-cell response of this individual is directed predominantly against a species-specific determinant(s) in *Der f* II. Whether or not *Der f* II, as opposed to *Der f* I for example, is an immunodominant T-cell allergen for the haplotype of this individual, such as has been described for the cytotoxic T-cell response to the internal components of influenza (Gotch *et al.*, 1987) warrants investigation. Alternatively, as suggested by the polyclonal response, T-cell recognition is neither limited to species-specific determinants, or indeed to *Der f* II, and

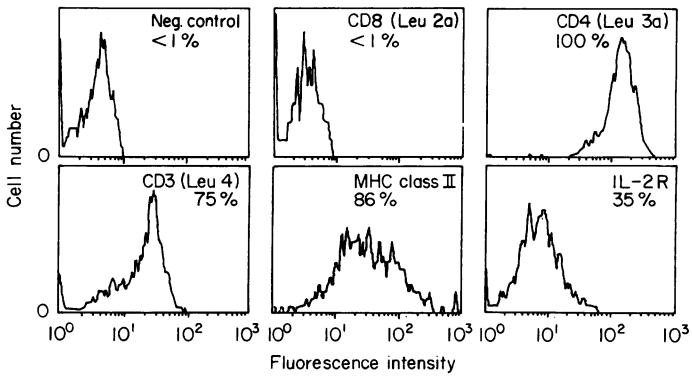

Figure 5. Indirect immunofluorescent cytofluorimetric analysis of clone DD11. Samples were analysed with monoclonal antibodies as indicated above the histograms. Percentage positivity is shown.

therefore the clonal response merely reflects the restriction element usage for a specific epitope.

In analysing the specificity of the *D. far.* IgE antibody response, similar to the T cells, recognition was predominantly of a protein that migrates with the same molecular weight as the *Der f* II allergen (12,500), although antibodies reactive with determinants that migrate with identical molecular weights to the f I and f III allergens (26,000 and 29,000) respectively (Heymann *et al.*, 1987) were also observed. These results are interesting for they suggest that while the T-cell recognition is mainly of the *Der f* II, allergen, the IgE antibody response is directed towards both the major and minor allergens. The human IgE antibody to *Der p* I and *Der f* I appears to be 80–95% cross-reactive (Chapman *et al.*, 1987), therefore it is possible that T cells primed to *D. pter.* are providing the helper activity for the cross-reactive B-cell response. Indeed, the PBMC of this individual were able to respond to *D. pter.* Whether or not the antibody response to *Der f* II and *Der f* III operates through the same mechanism is unclear. Alternatively, the *Der f* II-reactive T cells may provide not only specific helper activity for the anti-*f* II antibody response, but also 'intermolecular' help (Lake & Mitchison, 1977) for the anti-*f* I and *f* III B-cell response. These same arguments may apply to the immune response to *D. pter.* Furthermore, the fact that the cloned T cells and the line described here express membrane CD4 raises the possibility that they are able to regulate IgE synthesis, and therefore may be important in the pathogenesis of the allergic response to the house dust mite.

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