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Peptide specificity and HLA restriction do not dictate lymphokine production by allergen-specific T-lymphocyte clones

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SUMMARY

Human and murine $CD4^+$ T lymphocytes can be subdivided into distinct subsets [T-helper type 0 (Th0), Th1 or Th2], based on their lymphokine production profiles. Not much is known about the factors that determine these restricted lymphokine secretion profiles. Peptide specificity and human leucocyte antigen (HLA) restriction may be such factors. As it is well established that allergen-specific T lymphocytes from atopic individuals and non-atopic controls differ in their lymphokine secretion profile, we studied two allergen-specific T-lymphocyte clones (TLC) with identical peptide specificity and HLA restriction that were generated from the peripheral blood of an atopic donor and a non-atopic control donor. The two $CD4^+$ TLC recognize the same epitope (20–33) of the house dust mite *Dermatophagoides pteronyssinus* major allergen *Der p* II. Both TLC recognize the epitope in an HLA-DQB1*0602-restricted manner. However, the lymphokine production profiles of these TLC show clear differences after allergen-specific or polyclonal activation. As expected, TLC JBD4 from the atopic donor produced high levels of interleukin-4 (IL-4) without detectable interferon-γ (IFN-γ), whereas TLC PBA1 from the non-atopic donor produced both IFN-γ and IL-4 upon allergen-specific or polyclonal activation. Inasmuch as both TLC recognized the same epitope of *Der p* II in association with the same HLA-DQ molecule, these data suggest that peptide specificity and HLA restriction of human allergen-specific TLC do not dictate their lymphokine secretion profile.

INTRODUCTION

Allergic disease is characterized by high serum levels of allergen-specific IgE and by eosinophilia. These features are associated with the occurrence of allergen-specific type 2 T-helper cell (Th2)-like T lymphocytes that produce high levels of interleukin-4 (IL-4) and IL-5, but no or low amounts of IL-2 and interferon-γ (IFN-γ).[1–3] The presence of these Th2 cells has been demonstrated in affected tissues such as the skin, lungs and nasal mucosa.[4–8] The regulation of IgE production is under the control of several T-cell-derived lymphokines. IL-4 and IL-13 induce IgE production, whereas IFN-γ, TGF-β and TNF-α down-regulate IgE production.[9–12] IL-5 has been shown to play an important role in the differentiation of eosinophils.[13] As a consequence, allergen-specific Th2-like T lymphocytes that produce large amounts of both IL-4 and IL-5, but little or no IL-2 and IFN-γ, play an important role in the pathophysiology of atopic disease. House dust mite *Dermatophagoides pteronyssinus* (Dp) is an important source of environmental inhalation allergens. Based on IgE-binding studies, at least three major allergens (*Der p* I, *Der p* II and *Der p* III) and several minor allergens have been described.[14–17] At least two of these major allergens, *Der p* I and *Der p* II, are also important in the activation of T cells.[2,18–21] The genes encoding these major allergens have been cloned and sequenced,[22,23] enabling us to make synthetic peptides to identify the T-cell epitopes of these allergens. *Der p* I[21] and *Der p* II,[20,24] as well as other allergens studied to date,[25,26] appeared to contain multiple T-cell epitopes that are scattered throughout the molecule. Dp-specific TLC may be restricted by HLA-DRB1, HLA-DRB3, HLA-DQ (this paper) or HLA-DP gene products,[2,20,21,27] adding to the diversity of Dp-specific T cells. The amino acid sequence of peptides, and HLA restriction may be important factors that determine lymphokine production profiles of responding T cells.[28,29] We decided to study the IL-4 and IFN-γ production capacity of two *Der p* II-specific T-lymphocyte clones (TLC), one from an atopic donor and the other from a non-atopic control individual, with identical epitope specificity and HLA restriction.

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Abbreviations: Dp, *Dermatophagoides pteronyssinus*; Th, T-helper; TLC, T-lymphocyte clone.

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MATERIALS AND METHODS

Donors

Donor H (HLA DR2,11, DRw52, DQw1,w3) is an atopic asthma patient with elevated serum IgE levels (470 IU/ml) who is clinically allergic to Dp. Radioallergosorbent tests indicated high titres of total Dp and *Der p* II-specific IgE in the serum of this patient. Donor M (HLA-DR1,2, DQw1) is a non-atopic healthy control donor with a normal serum IgE level (< 10 IU/ml IgE) that has no Dp-specific IgE in the serum as indicated by radioallergosorbent tests.

Antibodies

Monoclonal antibodies (mAb) used for typing CD3 (OKT3)-, CD4 (OKT4)-, and CD8 (OKT8)-expressing cells were obtained from Ortho (Raritan, NJ). Subclasses of HLA class II restriction determinants were characterized by blocking antigen-specific T-cell proliferation with the following mAb: anti-HLA-DR (B.8.11.2, a gift from Dr F. Koning, Department of Immunohematology, University of Leiden, the Netherlands), anti-HLA-DQ (SPV L3–8; a gift from Dr H. Spits, Netherlands Cancer Institute, Amsterdam, the Netherlands), and anti-HLA-DP (B21/7, Becton Dickinson, Mountain View, CA).

Culture medium and cell culturing

T cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Gibco, Paisley, U.K.), supplemented with 10% pooled, c-inactivated normal human serum (Central Laboratory of the Blood Transfusion Service, Amsterdam, the Netherlands), gentamycin (80 mg/l) (Duchefa, Haarlem, the Netherlands) and, where mentioned, 10 U/ml recombinant rIL-2 (Cetus, Emeryville, CA). Epstein–Barr virus (EBV)-transformed B-cell lines were maintained in IMDM supplemented with 10% fetal calf serum (FCS) (Hyclone, Logan, UT) and gentamycin (80 μg/l). All cultures were incubated at 37° in a humidified atmosphere containing 5% CO^2 using tissue culture plates (Costar, Cambridge, MA).

Dp protein extract and purified Der p *II*

Protein extracts of Dp were prepared from lyophilized mite cultures (kindly provided by HAL, Haarlem, the Netherlands). After dialysis with phosphate-buffered saline (PBS), the HAL Dp extract used in this study contained $3{\cdot}75 \times 10^6$ U/ml *Der p* I and $38{\cdot}3 \times 10^6$ U/ml *Der p* II, as compared with the International Reference Preparation for Dp extracts (NIBSC 82/518).[2] *Der p* II was affinity purified using an anti-*Der p* II mAb as described in detail elsewhere.[15] The final preparation contained $8{\cdot}5 \times 10^6$ U/ml *Der p* II and 160 U/ml *Der p* I. Recombinant *Der p* II^{23} was prepared by G. Hakkaart (Central Laboratory of the Blood Transfusion Service).

Preparation of synthetic Der p *II peptides*

The preparation of the synthetic peptides used in this study is described in detail elsewhere.[20,30]

Cloning procedure of Der p *II-specific T cells*

Der p II-specific T cells were cloned from short-term *Der p* II-reactive T-cell lines using a protocol as described previously.[1,20] Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Lymphoprep (Nycomed, Torshov, Norway) and cultured for 11 days in the presence of affinity-purified *Der p* II (5 μg/ml), the last 5 days in the presence of 10 U/ml rIL-2. On day 11, cloning was performed in 96-well flat-bottom culture plates, by limiting dilution at 0·3 cells/well and non-specific stimulation with 1 μg/ml phytohaemagglutinin (PHA) (Difco, Detroit, MI) in the presence of a feeder mixture of 3000 rads-irradiated PBMC (10^5/well) from two different unrelated donors, 3000 rads-irradiated cells (10^4/well) of an EBV-transformed B-cell line (JY) and rIL-2 (10 U/ml). After about 2 weeks, expanding clones were transferred to 24-well plates in culture medium supplemented with rIL-2 (10 U/ml). After an additional 5–10 days, most clones had produced enough cells to test for Dp specificity in a proliferation assay. *Der p* II-reactive TLC were further expanded by restimulation with PHA ($2{\cdot}5 \times 10^5$ cells/well) in 24-well plates, and maintained in the same feeder mixture as described above.

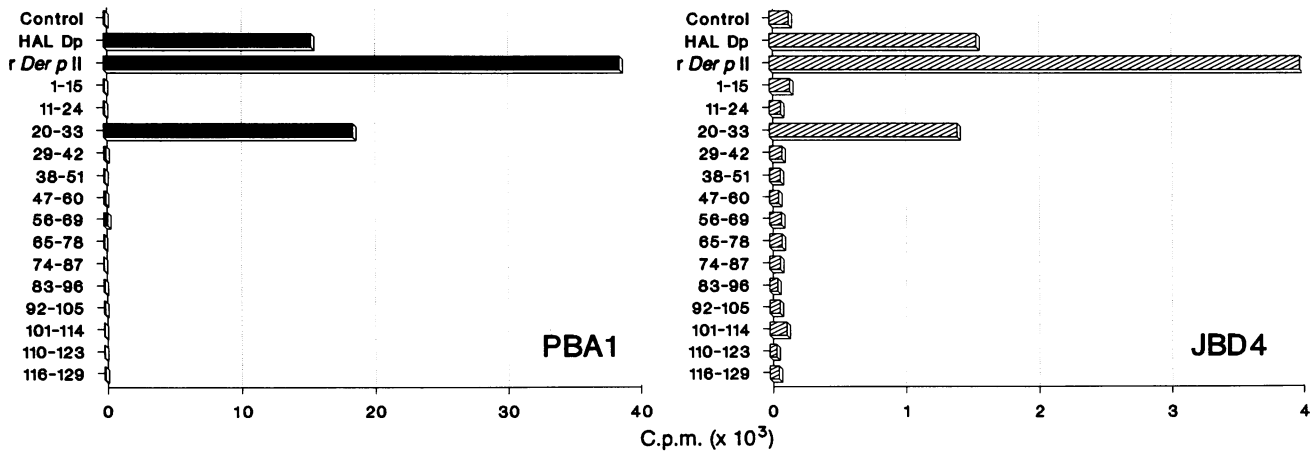

Figure 1. Determination of T-cell epitope specificity of *Der p* II-specific TLC PBA1 of non-atopic donor M and JBD4 of atopic donor H. TLC ($2–4 \times 10^4$/well) were tested for their proliferative response in the presence of autologous PBMC (10^5/well) to all 14 synthetic peptides of *Der p* II, r*Der p* II and a total Dp protein extract. Values given are c.p.m. as determined by [^{3}H]TdR incorporation. Data represent one out of two such experiments.

Table 1. HLA-DQ restriction of TLC PBA1 and JBD4

	Stimulation index (SI)*				
	Dp	Dp + anti-HLA-DP	Dp + anti-HLA-DQ	Dp + anti-HLA-DR	% inhibition† anti-HLA-DQ
PBA1	19·1	36·5	0·7	16·1	100%
JBD4	27·9	70·4	0·4	104·1	100%

*TLC PBA1 and JBD4 ($2–4 \times 10^4$/well) were stimulated with Dp protein extract in the presence of autologous PBMC (10^5/well) as APC. Anti-HLA class II mAb were added to suppress Ag presentation to the TLC. Representative data from one out of three similar experiments are shown.

† The suppressive effect of anti-HLA-DQ mAb is expressed as follows (NB in all values background proliferation is subtracted):

$$\% \text{ inhibition} = \frac{(\text{c.p.m.} - \text{mAb}) - (\text{c.p.m.} + \text{mAb})}{(\text{c.p.m.} - \text{mAb})} \times 100\%$$

Fresh rIL-2 was added at 3-day intervals. Fresh feeder cells and PHA were added every 14 days.

Cell typing

Cell typing was performed by labelling suspended cells with surface antigen (Ag) specific mouse mAb and detecting positive cells by fluorescence microscopy, or by flow cytometry (FACScan, Becton Dickinson) using fluorescence isothiocyanate (FITC) conjugated $F(ab')_2$ fragments of a rabbit anti-mouse antiserum (Zymed, San Francisco, CA).

Ag-specific T-cell proliferation assays

Ten to fourteen days after the last stimulation, T cells were washed three times in Hanks' balanced salt solution (HBSS) + 2% FCS to remove all rIL-2. Subsequently, Ag-specific proliferation of the TLC was assayed by culturing the cells ($2–4 \times 10^4$/well) in 96-well flat-bottom culture plates in the presence of specific Ag, using 3000 rads-irradiated autologous PBMC (10^5/well) or EBV-transformed B cells (10^4/well) as antigen presenting cells (APC). Cells were cultured for 40 hr, the last 16 hr in the presence of 0·3 μCi (= 11·1 kBq)/well of [^{3}H]TdR (Amersham International, Amersham, UK). Proliferation was expressed as the mean c.p.m. of duplicate cultures, except when mentioned otherwise. *Der p* II-specific proliferation of T cells in PBMC ($1–2 \times 10^5$/well) was assayed after 6 days of culture in the presence of affinity-purified *Der p* II (5 μg/ml), also terminated by a 16-hr [^{3}H]TdR pulse. Proliferation was expressed as the mean c.p.m. of triplicate cultures.

Production of TLC supernatants for cytokine measurements

Ten to fourteen days after the last stimulation T cells were washed three times in HBSS + 2% FCS and stimulated in 96-well tissue culture plates at 4×10^4 cells/well. T-cell supernatants were obtained by incubating the TLC for 24 hr in the presence of a mitogenic pair of mAb directed against CD2 (CLB-T11.1/1 and CLB-T11.2/1; 1:1000 dilution of ascites) in combination with a mAb directed against CD28 (CLB-28/1; 1:1000 dilution of ascites) and phorbol 12-myristate 13-acetate (PMA). Alternatively, we collected supernatants of antigen-specific stimulated TLC cells before adding [^{3}H]TdR at 24 hr. For standardization purposes both polyclonal and antigen-specific supernatants were collected at the 24-hr time-point.

IL-4 and IFN-γ assays

Estimation of the IL-4 and IFN-γ contents of TLC supernatants was performed with specific solid-phase sandwich ELISA as described in detail elsewhere.[31,32]

RESULTS

Characterization of *Der p* II-specific TLC

Short-term *Der p* II-specific T-cell lines were generated from the peripheral blood of atopic donor H and non-atopic control donor M. Two *Der p* II-specific TLC were generated from these lines. Both TLC express CD3 and CD4, but not CD8 (data not

Table 2. Reactivity of TLC PBA1 and JBD4 with several APC

				Proliferation (c.p.m. × 10^{-3})*			
	HLA type			PBA1		JBD4	
Donor	DRB1	DRB3	DQ	—	Dp	—	Dp
W	11, w9	w52, w53	w3	0·1	0·2	0·1	0·1
H	4, w9	w53	w3	0·1	0·2	0·1	0·1
M	4, 11	w52, w53	w3	0·1	0·0	0·1	0·1
L	1, 3	w52	w1, w2	0·0	0·0	0·1	0·1
K	1, 3	w52	w1, w2	0·1	0·1	0·1	0·1
D	13, 14	w52	w1	0·3	9·9	0·1	0·1
G	2, w6	w52	w1	0·1	42·3	0·1	20·7
S	1, 2	—	w1	0·1	23·6	0·1	2·7
MM	1, 2	—	w1	0·1	66·6	0·1	4·9
KH	2, 11	w52	w1, w3	0·0	36·4	0·1	8·7

**Der p* II-specific TLC PBA1 and JBD4 ($2–4 \times 10^4$/well) were stimulated with Dp protein extract in the presence of 10 differently tissue-typed PBMC (10^5/well) as APC. Data are shown as the mean c.p.m. of triplicate cultures as determined by [^{3}H]TdR incorporation. Data represent one of three such experiments.

Table 3. Lymphokine production of TLC PBA1 and JBD4*

TLC	Stimulation	c.p.m. ($\times 10^{-3}$)	IL-4 (ng/ml)	IFN-γ (ng/ml)	Ratio IL-4/IFN-γ
PBA1	r*Der p* II†	70·7	0·8	1·8	0·4
	αCD2/CD28 + PMA‡	63·4	2·5	8·5	0·3
JBD4	r*Der p* II	44·5	3.2	<0·1	>32·0
	αCD2/CD28 + PMA	61·2	3·3	0·6	>5·5

* Data shown are representative of six similar measurements.

† TLC (2–4 × 10^4/well) were stimulated with Dp protein extract in the presence of autologous PBMC (10^5/well). Supernatants were collected after a 24-hr culture period.

‡ TLC (10^5/well) were stimulated by incubation with a mitogenic pair of αCD2 mAb and an αCD28 mAb in the presence of PMA (1 ng/ml). Supernatants were collected after a 24-hr culture period.

shown). Using a panel of overlapping synthetic peptides of *Der p* II, the TLC were found to recognize peptide 20–33 of the *Der p* II molecule (Fig. 1).

HLA restriction of *Der p* II-specific TLC

Proliferation experiments using anti-HLA-DR, -DP and -DQ mAb were performed to identify the HLA class II restriction of the TLC. As is shown in Table 1, the proliferative response to Dp extract of both clones was completely inhibited after the addition of anti-HLA-DQ mAb, whereas addition of anti-HLA-DR and anti-HLA-DP mAb had no effect. Additional experiments showed that the mAb SPV-L3 was not cytotoxic and inhibited Ag-specific proliferation completely up to a dilution factor of 10,000 of ascites (data not shown). To determine the exact HLA-DQ gene product involved, proliferative responses to Dp were measured in the presence of PBMC from 10 different HLA-typed donors. Table 2 shows that both clones could recognize *Der p* II only if presented by PBMC of donors expressing HLA-DQw1, especially when they expressed both HLA-DR2 and HLA-DQw1, indicating that the TLC were restricted by the HLA-DQB1*0602 allele, a subtype of HLA-DQw1 which is in linkage disequilibrium with HLA-DR2. This was confirmed by typing of HLA-DQw1 using allele-specific oligonucleotides of the donors of both TLC (data not shown).

IL-4 and IFN-γ production

Clear differences were found in lymphokine profile between the two otherwise very similar TLC (Table 3). TLC JBD4 of atopic donor H produces large amounts of IL-4, but no detectable IFN-γ after both antigen-specific as well as polyclonal activation. TLC PBA1 of non-atopic donor M produces both IFN-γ and IL-4 after both modes of stimulation. The production patterns remained stable after repeated stimulations.

DISCUSSION

The data presented here clearly show that peptide specificty and HLA restriction of allergen-specific TLC do not dictate their lymphokine production capacity. Previous studies, however, suggested that epitope specificity and HLA restriction may be important factors influencing lymphokine production patterns by T lymphocytes.[28,29] To investigate the relevance of these findings in atopic disease, in which lymphokine production patterns of allergen-specific T cells appear to be very important for the pathophysiology of the disease, we studied the possible correlation between epitope specificity, HLA restriction and lymphokine production. To this aim, two *Der p* II-specific TLC were generated that recognized the same peptide (20–33) of the allergen in the context of the same HLA class II molecule (HLA-DQB1*0602). The finding that two TLC with the same peptide specificity have the same HLA restriction is consistent with the findings of IJssel *et. al.* who described several T-cell epitopes of the allergen *Der p* I that seemed to correlate with HLA restriction.[21] It should be noted, however, that it has also been shown that the same epitope may be recognized by T cells of donors with completely different HLA type, and that the same HLA molecule may present different peptides of the same allergen,[20,21,24,26] indicating that peptide 20–33 may also be presented by other HLA molecules. Indeed we have identified reactivity with this peptide in two other patients with different HLA type.[20]

Clear differences were found in lymphokine profile between the two otherwise very similar TLC. TLC JBD4 of atopic donor H produces large amounts of IL-4, but no detectable IFN-γ, whereas TLC PBA1 of non-atopic donor M produces both IFN-γ and IL-4. These data suggest that peptide specificity and HLA restriction do not dictate the lymphokine secretion profile of allergen-specifc T lymphocytes. In addition, the present data agree with previous reports that show that the differentiation and outgrowth of Th0, Th1 and Th2 cells are under the control of exogenous factors such as IL-4,[33,34] IFN-γ,[33] IL-12,[35] and prostaglandin E_2 (PGE_2).[36] This has been confirmed in animal models using T-cell receptor transgenic mice.[37,38] Therefore, our data may give additional evidence for the hypothesis that functional maturation of $CD4^+$ T lymphocytes is influenced by environmental factors, such as lymphokines and mediators, and not by epitope specificity and HLA restriction.

Taken together, the present data imply that no strict correlation exists between T-cell specificity and lymphokine production, and that the factor(s) that determine the outgrowth of naive Th cells into memory Th cells have to be studied in more detail, in order to understand the underlying causes of atopic disease.

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