Allergens, IgE, mediators, inflammatory mechanisms

Parietaria judaica–specific T-cell clones from atopic patients: Heterogeneity in restriction, Vβ usage, and cytokine profile

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The pollen of Parietaria *spp. is one of the most clinically relevant sources of allergens in the Mediterranean area. CD4+ T-lymphocyte clones specific for* Parietaria *allergens were isolated from peripheral blood of atopic donors, and their phenotype, HLA restriction, Vβ usage, and cytokine profile were determined. All the T-cell clones expressed the α/β T-cell receptor and were induced to express CD40 ligand after activation with phorbol-myristate-acetate plus ionomycin. When the proliferative response to three chromatographic fractions of the extract was analyzed, distinct reactivity patterns were found. Interestingly, most of the clones responded to the fraction that was the most enriched for the major allergen Par j 1. The clones were either HLA-DR– or HLA-DQ–restricted and did not show any preferential usage of T-cell receptor Vβ segments. Five of the 17 clones tested produced only IL-4 and no interferon-γ, thus displaying a T_{H2} phenotype. The other clones displayed a T_{H0} phenotype in that they produced both IL-4 and interferon-γ. These results show that in atopic patients T-cell response against* Parietaria judaica *allergen involves different T-cell subsets in terms of restriction, Vβ usage, and cytokine profile. (J ALLERGY CLIN IMMUNOL 1996;97:627-37.)*

Key words: Parietaria judaica, *allergens, T-cell clones, T_{H1}, T_{H2}, HLA restriction, Vβ usage, cytokines, IL-4, IFN-γ*

The regulatory role of $CD4^+$ T lymphocytes on the IgE antibody production by B cells in response to environmental allergens is well documented.[1, 2] $CD4^+$ helper T lymphocytes have been divided into T_{H1} and T_{H2} cells, or the intermediate T_{H0}, on the basis of their lymphokine-producing profiles.[3-5] The reciprocal activity on IgE production of interferon-γ (IFN-γ) and IL-4, produced respectively by T_{H1} and T_{H2} cells, has been studied both in mice and human beings.[6, 7] IL-4 has been shown to act as a switching factor in inducing IgE synthesis by B lymphocytes, whereas IFN-γ downregulates IgE production.[8, 9] IL-5, which is also produced by T_{H2} cells, is another key factor in allergic disease through its regulatory action on eosinophil maturation, survival, and activation[10] and on basophil functions.[11] T_{H2} cells are therefore essential to

Abbreviations used

EBV:	Epstein-Barr virus
FITC:	Fluoroscein isothiocyanate
IFN:	Interferon
mAb:	Monoclonal antibody
PBMCs:	Peripheral blood mononuclear cells
PBST:	Phosphate-buffered saline–Tween 20 (0.05% vol/vol, pH 7.2)
PHA:	Phytohemagglutinin
PjE:	*Parietaria judaica* pollen extract
PMA:	Phorbol-myristate-acetate
PPT:	$(NH_4)_2\ SO_4$ precipitate
SDS-PAGE:	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TCR:	T-cell receptor
TLC:	T-lymphocyte clone

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the IgE response and play an important role in the pathophysiology of allergic disorders through the secretion of IL-4 and IL-5. Moreover, IL-10 and IL-13, produced by different cell types including T_{H2}, are involved in the regulation of allergen-specific IgE response through their effects on T_{H1} and B cells, respectively.[12, 13]

T-cell response has been studied at the clonal level against different allergens, mainly proteins of house dust mite,[1, 14-18] Hymenoptera venoms,[19, 20] animal dander,[21] and pollens from Graminaceae,[2, 22, 23] Compositae,[24] and Betulaceae families.[25]

The pollen of *Parietaria* spp. is one of the most clinically relevant sources of allergens in the Mediterranean area and is responsible for almost 30% of atopic respiratory tract diseases.[26] In previous studies we investigated the T-lymphocyte proliferative response to an allergenic extract of *Parietaria judaica*[27] (PjE) and to its separated fractions.[28]

In this study we have isolated and characterized a panel of human T-lymphocyte clones (TLCs) specific for PjE from peripheral blood of five subjects allergic to *Parietaria* spp. only. These clones express different Vβ gene products, are either HLA-DR– or HLA-DQ–restricted, and display a T_{H2} or T_{H0} phenotype on the basis of their lymphokine production profiles.

METHODS

P. judaica protein preparations

PjE. P. judaica pollen was purchased from Allergon (Angelholm, Sweden). The whole pollen extract (PjE) was obtained as reported for *Parietaria officinalis.*[29] The protein content, measured in the lyophilized preparation according to the method of Bradford,[30] was approximately 25%. After being reconstituted in culture medium, the extract was filtered through a Millex-GV 0.22 μm Filter Unit (Millipore S.A., Malsheim, France).

Chromatographic enrichment of P. judaica *major allergen (Par j 1).* Ammonium sulfate to 65% saturation was slowly added to 400 mg of the lyophilyzed PjE dissolved in 100 ml of 125 mmol/L NH_4HCO_3 (concentration, 1 mg/ml proteins); after overnight stirring at 4° C, the suspension was centrifuged at 4° C (10,500 *g* for 30 minutes), and the pellet was resuspended in 125 mmol/L NH_4HCO_3. After extensive dialysis against 125 mmol/L NH_4HCO_3 and then twice-distilled water, the precipitate (65% $(NH_4)_2SO_4$ precipitate [PPT]) was lyophilyzed and stored at 4° C under vacuum. The supernatant was applied to a 2.5 × 10 cm Octyl-Sepharose CL4B (Pharmacia-LKB, Uppsala, Sweden) column, previously equilibrated in 125 mmol/L NH_4HCO_3/65% $(NH_4)_2SO_4$, at a flow rate of 3 ml/hr. After the effluent was collected, the column was washed in 125 mmol/L NH_4HCO_3/65% $(NH_4)_2SO_4$, then eluted step by step under continuous monitoring at 280 nm with the following conditions: 125 mmol/L NH_4HCO_3/32.5% $(NH_4)_2SO_4$ (eluate 1), 125 mmol/L NH_4HCO_3 (eluate 2), 125 mmol/L NH_4HCO_3/50% ethylene glycol (eluate 3). The effluent and the three eluates were dialyzed against water and lyophilized. Protein content of the fractions was determined according to the method of Bradford.[30]

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and IgE immunoblotting

SDS-PAGE[31] was carried out in a refrigerated system at 8° C (Mini Protean II Cell; Bio-Rad, Richmond, Calif.) under constant current (8 mA in the stacking gel and 15 mA in the separating gel). PjE and its chromatographic fractions (10 μg protein/well) were reduced by 5% vol/vol 2-mercaptoethanol before they were applied to the 15% wt/vol polyacrylamide gel. Immunoblotting was performed in a Bio-Rad apparatus after SDS-PAGE separation. The blotted nitrocellulose strips were incubated overnight with 1 ml of pooled human sera from 10 subjects allergic to only *Parietaria* spp., diluted 1:2 in phosphate-buffered saline–Tween 20 (0.05% vol/vol, pH 7.2) (PBST), at room temperature, washed five times with PBST, and then incubated overnight with iodine 125–labeled rabbit anti-human IgE (RAST I, Pharmacia) (approximately 100,000 cpm/strip) diluted in PBST. The strips were then washed five times, and the reactive bands were detected by autoradiography at −80° C for 3 days with the use of x-ray film (Kodak Diagnostic Film X-Omat AR; Eastman Kodak Company, Rochester, N.Y.).

Donors

Five atopic patients were selected for this study (age range, 21 to 41 years) after informed consent was obtained. These subjects, who had never received specific immunotherapy, were diagnosed as being allergic to *Parietaria* pollen only on the basis of a clinical history of rhinitis and bronchial asthma and positive results of skin prick test and RAST (Pharmacia), performed with a panel of common allergens.

Medium for cell culturing

The medium used throughout was RPMI 1640 medium (Dutch modification; ICN Flow, Irvine, U.K.), supplemented with 5% pooled normal human serum (Netherlands Red Cross, Amsterdam, Netherlands), 2 mmol/L L-glutamine (ICN Flow), 50 μg/ml kanamycin (Sigma Chemical Co., St. Louis, Mo.), 1 mmol/L sodium pyruvate (ICN Flow), 1% nonessential amino acids (Sigma Chemical Co.), 50 nmol/L 2-mercaptoethanol (Merck, Darmstadt, Germany) (referred to as complete medium).

Isolation of allergen-specific TLCs

Peripheral blood mononuclear cells (PBMCs) obtained by the standard Ficoll-Paque method (Pharmacia) were cultured in 96-well flat-bottomed plates (Costar, Cambridge, Mass.) in complete medium at

TABLE I. Proliferation of TLCs against three different concentrations of PjE

Exp. 1				Exp. 2			
Clone	50 μg/ml	5 μg/ml	0.5 μg/ml	Clone	50 μg/ml	5 μg/ml	0.5 μg/ml
1A4	113.0	178.1	110.7	2A3	16.3	6.7	ND
1A5	18.0	8.1	ND	2A5	52.4	18.0	3.7
1A6	54.3	5.4	0	2A8	36.2	10.4	0
1B2	35.7	3.9	ND	2B2	38.7	36.0	16.3
1B3	10.2	7.1	ND	2B12	18.1	13.7	ND
1B6	96.9	20.1	ND	2C3	36.2	31.2	15.7
1B12	41.2	2.8	0	2C6	32.7	2.6	0
1C1	13.1	0.6	0	2C11	26.3	17.8	ND
1C3	19.8	19.2	0.3	2D2	53.0	21.8	ND
1C6	21.6	7.3	ND	2D4	146.7	142.8	78.8
1C10	15.5	13.5	1.3	2D5	13.5	6.6	0
1C11	17.3	0.3	0	2D6	10.8	8.4	12.8
1C12	10.0	9.8	0	2D8	39.4	1.2	0.9
1D3	18.7	11.4	3.6	2D12	9.7	7.3	ND
1D9	19.2	1.0	0	2E4	10.2	4.6	0
1D11	44.4	2.7	0.2				
1E2	31.7	4.5	ND				
1E3	48.4	38.1	14.6				
1E5	37.7	7.2	ND				
1E7	7.5	1.3	0				
1E8	16.7	3.9	ND				
1E12	73.7	57.4	30.2				
1F2	19.8	8.6	ND				
1F5	25.5	1.3	ND				
1F8	24.3	3.4	0				

Responses are expressed as counts per minute $\times 10^{-3}$, subtracted out of the background responses without antigen.
ND, Not done.

37° C in a humidified atmosphere containing 5% CO_2. PjE (50 μg protein/ml) was added on day 0 and rIL-2 (20 U/ml; PeproTech, Rocky Hill, N.J.) was added on days 5 and 9. After 12 days, growing lines were tested for antigen specificity in a proliferation assay with PjE. Allergen-specific TLCs were obtained from these short-term PjE-reactive T-cell lines by limiting dilution. Viable T-cell blasts enriched on Ficoll gradient were cloned at 0.3 cells/well in 96-well round-bottomed plates (Costar) in a volume of 200 μl in the presence of 10^5 feeder cells (5000 rad-irradiated PBMCs from unrelated donors), 1 μg/ml phytohemagglutinin (PHA) (ICN Flow), and 30 U/ml rIL-2. After 2 weeks, growing microcultures were tested for PjE specificity in a proliferation assay, and PjE-reactive clones were expanded in 24-well plates (Costar) and maintained in rIL-2–containing complete medium. Feeder cells (5×10^5/well) were added at 20-day intervals together with 1 μg/ml PHA. TLCs were always used 10 to 15 days after restimulation.

Epstein-Barr virus (EBV) transformation of B cells

Autologous EBV–B-cell lines were prepared from PBMCs of the TLC donors by in vitro infection with EBV obtained from the supernatant of the EBV-producing marmoset line B95/8 (ATCC, Rockville, Md.), in the presence of 2 μg/ml Cyclosporin A (Sandoz, Basel, Switzerland). The cells were maintained in complete medium in which normal human serum was replaced by 10% fetal calf serum (ICN Flow).

Antigen-specific T-cell proliferation assays

T-cell line or TLC cells (2.5×10^4) were cultured in complete medium in 96-well flat-bottomed plates with an equal number of autologous irradiated (10,000 rad) EBV-B cells for 48 hours in the presence of suitable concentrations of PjE or PjE-enriched fractions. Proliferation was measured by liquid scintillation in a Beckman β-counter after a 16-hour pulse with 1 μCi/well tritiated thymidine (Amersham Life Science, Buckingham, U.K.). The data were recorded as mean counts per minutes of triplicate cultures.

HLA class II restriction was determined by blocking dose-dependent antigen-specific proliferation of the TLCs with the following mouse monoclonal antibodies (mAbs): clone L243 (IgG_{2a}, anti-DR), clone B7.21 (IgG_1, anti-DP), clone SPVL3 (IgG_{2a}, anti-DQ), clone W6/32 (IgG_{2a}, anti-major histocompatibility complex

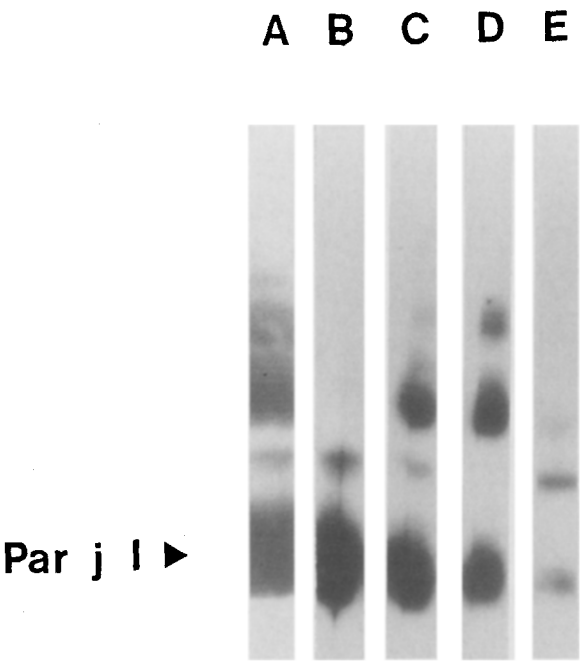

FIG. 1. Immunoblotting pattern of PjE and its chromatographic fractions, developed with specific human IgE. **A,** PjE; **B,** eluate 1; **C,** eluate 2; **D,** eluate 3; **E,** 65% PPT. The major allergen Par j 1 is indicated by the *arrow.*

class I; kindly provided by Dr. A. Lanzavecchia, Basel Institute for Immunology, Basel, Switzerland). The ascitic fluids were titrated in preliminary experiments in which twofold dilutions of the four mAbs, ranging from 1:250 to 1:2000, were added at the initiation of the proliferation culture with three different antigen doses. On the basis of the results obtained, the dilution of 1:500 for all four mAbs was chosen and used in all the subsequent experiments.

Phenotypic analysis

TLC cells were analyzed for expression of surface markers by flow cytometry with a FACScan (Becton Dickinson, Mountain View, Calif.). The following mAbs, labeled with fluoroscein isothiocyanate or phycoerythrin, were used, according to the manufacturer's instructions: anti-CD3 (Leu-4), anti-CD4 (Leu-3a), anti-CD8 (Leu-2a), anti-T-cell receptor (TCR) α/β, anti-CD45RA (Leu-18) (all purchased from Becton Dickinson), anti-CD45RO (UCHL1) (Dakopatts, Copenhagen, Denmark). A panel of 12 mAbs recognizing different Vβ gene products (Vβ2, Vβ3, Vβ5.2, Vβ5.3, Vβ8, Vβ11, Vβ13.3, Vβ16, Vβ17, Vβ19, Vβ21, and Vβ22 [kindly provided by Dr. A. Necker, Immunotech, Marseille, France]) was used, followed by staining with FITC-labeled goat anti-mouse antibodies (Southern Biotechnology Association, Birmingham, Ala.).

CD40L expression after activation

CD40L expression on TLCs was induced by activating 1×10^5 TLC cells with 10^{-7} mol/L phorbol-myristate-acetate (PMA, Sigma Chemical Co.) alone or with 1 μg/ml ionomycin in 96-well flat-bottomed plates for 8 hours at 37° C. Cells were then harvested and stained with an anti-CD4 mAb (clone 6D10) together with a CD40-human IgM fusion protein (kindly provided by Dr. P. Lane, Basel Institute for Immunology), followed by the appropriate second antibodies FITC- and phycoerythrin-labeled. An anti-CD69 mAb (kindly provided by Dr. R. Testi, University of Rome, Italy) was used as an activation control.

Stimulation of TLCs for cytokine production and cytokine detection assay

TLC cells (5×10^5) were cultured in 12 × 75 mm round-bottomed tubes (Costar) with 50 μg/ml PjE in the presence of 1×10^5 autologous 6000 rad-irradiated EBV-B cells as antigen-presenting cells for 60 hours, or with PMA (10^{-7} mmol/L) plus mAb anti-CD3 (200 ng/ml) without antigen-presenting cells for 40 hours. The cultures were performed in 0.5 ml complete medium with 10% fetal calf serum. Supernatants were harvested by centrifugation, aliquoted, and stored at −80° C before testing. Levels of IL-2, IL-4, and IFN-γ were measured by commercial sandwich ELISA (Quantikine; R&D Systems, Minneapolis, Minn.) according to the manufacturer's instructions. The detection limits of the three kits were 10 pg/ml, 10 pg/ml, and 20 pg/ml, respectively. Positivity criteria for IL-4 and IFN-γ production were defined as follows. Mean value of the amounts of cytokine plus 3 standard deviations of the mean, calculated for each tested cytokine in the supernatant of the unstimulated cultures of TLCs (spontaneous production), was regarded as the negativity cutoff. Thus all of the TLCs producing an amount of cytokine greater than 44 pg/ml for IL-4 and greater than 208 pg/ml for IFN-γ in the supernatant were scored as positive for the production of the cytokine.

RESULTS

Isolation of *Parietaria*-specific TLCs

Beginning with PBMCs from five different allergic subjects, short-term lines specific for PjE were established and cloned by limiting dilution. Different numbers of PjE-specific TLCs were obtained with a percentage, calculated from the total number of growing clones, ranging from 6% to 35%. Forty PjE-specific TLCs from two subjects (referred to as Exp. 1 and Exp. 2) were selected for further analysis. All clones were CD4+ CD8− CD45RO+, expressed the TCR α/β, and proliferated in response to increasing concentrations of PjE (Table I). These clones did not recognize non-cross-reactive extracts from other allergenic pollens (*Olea europaea* and *Cupressus arizonica,* data not shown).

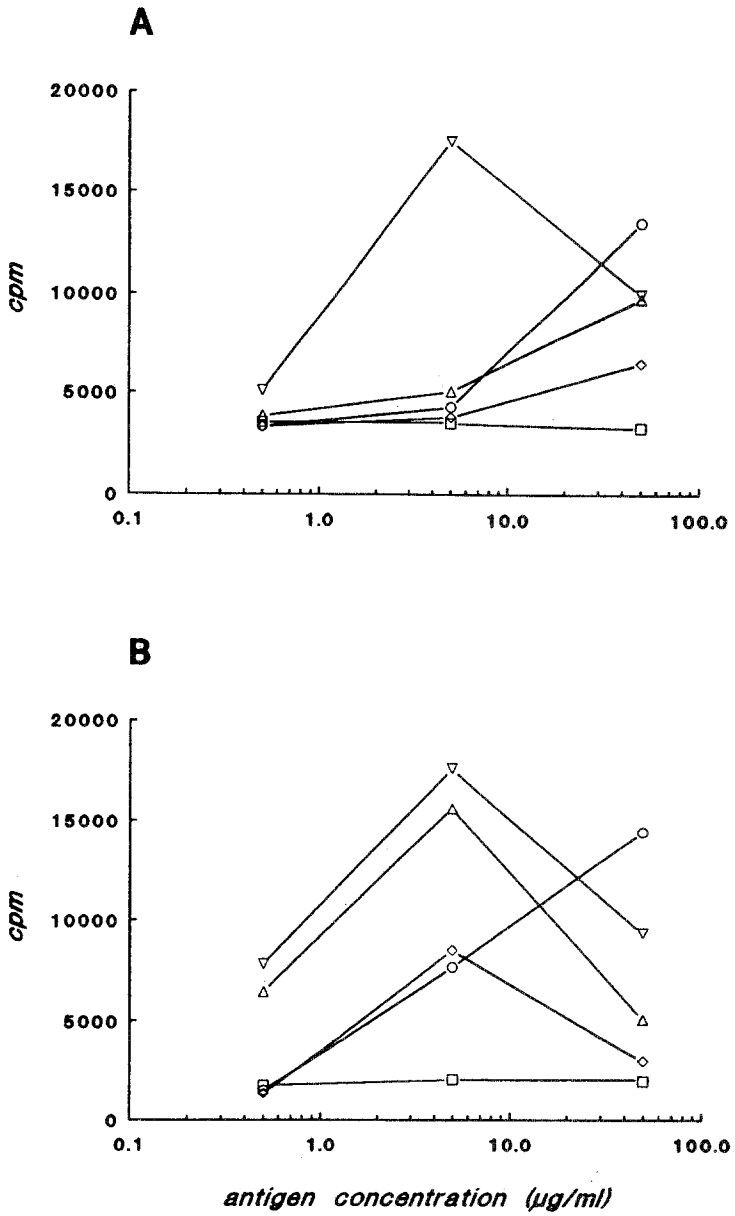

FIG. 2. Dose-dependent response of two representative TLCs, 1E8 **(A)** and 2D5 **(B)**, against the chromatographic fractions of PjE: ○, PjE; □, 65% PPT; ▽, eluate 1; △, eluate 2; ◇, eluate 3. The background response without antigen was 3118 cpm (1E8) and 1279 cpm (2D5).

Reactivity to *Parietaria* fractions

The IgE immunoblotting of the Octyl-Sepharose fractions after SDS-PAGE analysis (Fig. 1) indicated that the highest enrichment of the major allergen Par j I of *P. judaica* was found in eluate 1, whereas in the other two fractions, eluates 2 and 3, the selective loss of different PjE components was recorded. Sixty-five percent PPT lacked almost completely the major allergen. When these fractions were tested with the TLCs, the results, reported in Fig. 2 for two clones, showed that the TLCs had distinct reactivity patterns with the different fractions, although the majority of them showed a dose-dependent response to eluate 1 higher than the response to the other fractions tested, whereas 65% PPT was not able to induce a proliferative response in any of the TLCs, thus demonstrating that after the $(NH_4)_2SO_4$ precipitation of PjE, the T-cell–reactive components are essentially recovered in the supernatant. These data also demonstrate that the antigenic properties of PjE needed to induce a T-cell response are

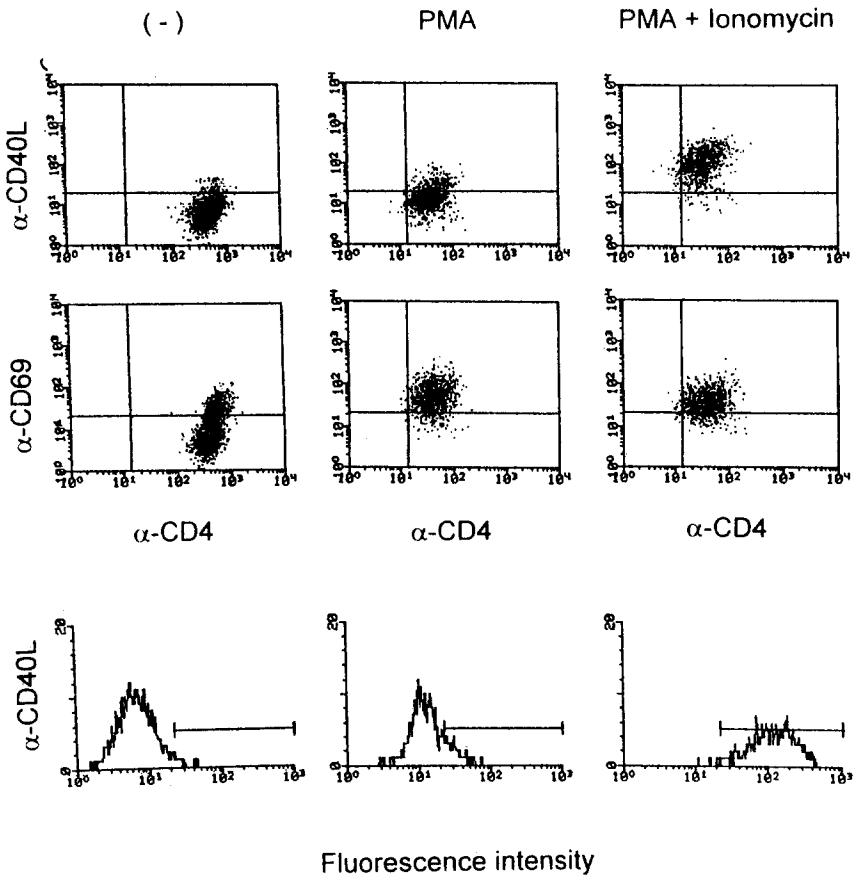

FIG. 3. FACS diagrams showing CD40L expression on a representative TLC after no activation *(left column)*, activation by PMA *(center column)*, and activation by both PMA and ionomycin *(right column)*. CD4 and CD69 expression were checked as control (see text).

TABLE II. Blocking of the dose-dependent proliferation of clone 2D4 by anti-DR mAb

mAb dilution	PjE 50 μg/ml	PjE 10 μg/ml	PjE 2 μg/ml
—	153,681	151,617	102,753
1:250	13,509	3,123	2,394
1:500	28,593	5,760	2,415
1:1000	51,027	22,374	3,282
1:2000	144,996	71,283	16,098

Responses are expressed as counts per minute. Background response without antigen was 2397 cpm.

maintained after the Octyl-Sepharose fractionation procedure.

CD40L expression after activation

To evaluate the ability of PjE-specific TLCs to express CD40L after activation, we incubated T cells alone or with PMA or PMA plus ionomycin for 8 hours and then evaluated the presence of CD40L with a soluble form of its ligand CD40. Fig. 3 shows the FACS diagrams obtained with a representative clone. In the absence of any stimulus the cells were CD40L-negative, and only in a small percentage CD69-positive, an activation marker used as control. After activation with either PMA or PMA plus ionomycin, the cells expressed CD69 and downregulated CD4. However, the expression of CD40L was detected only after induction with both stimuli. Similar results were obtained with all the clones tested.

HLA class II restriction and Vβ usage in the TCR

After preliminary titration experiments of the anti-HLA-class II mAbs (see Table II for representative experiments with the DR-restricted clone 2D4) in which the 1:500 dilution was chosen, the proliferation-blocking effects of the anti-HLA-DP, anti-HLA-DQ, and anti-HLA-DR mAbs were tested on a group of 14 TLCs stimulated with different doses of PjE to determine the HLA class II restriction of the TLCs.

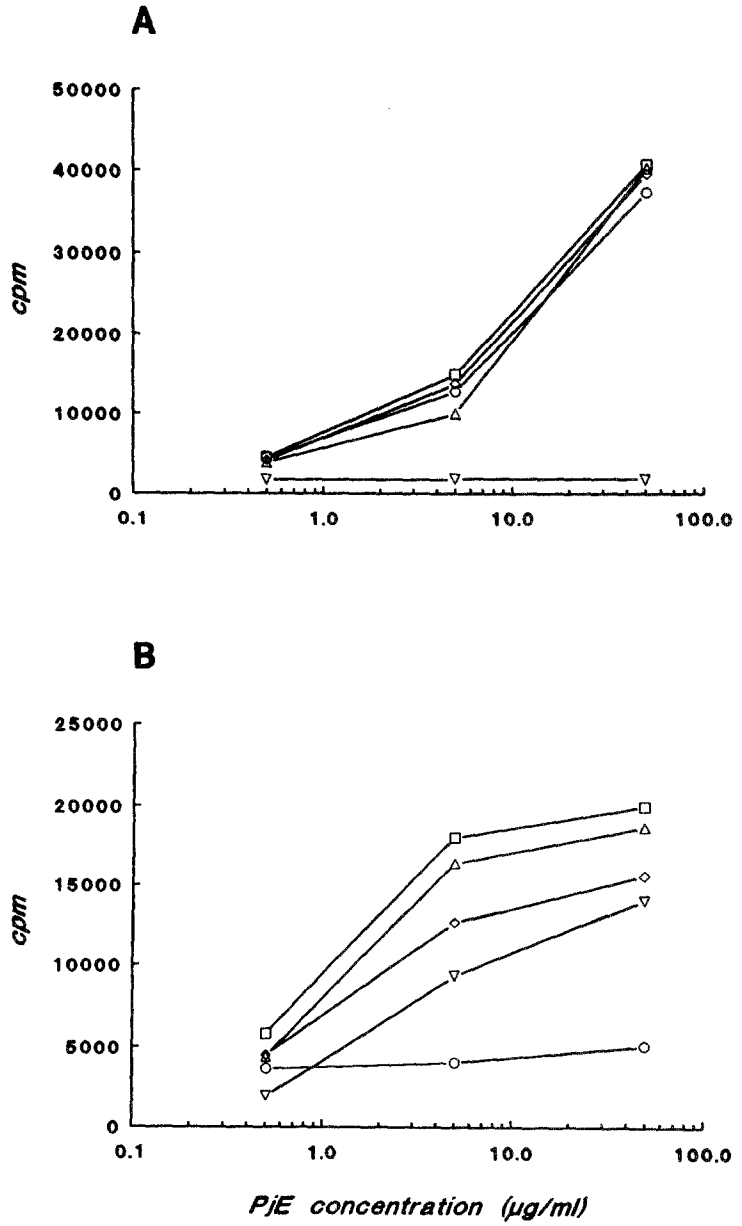

FIG. 4. Inhibition of PjE-induced proliferation of two representative TLCs, 1E3 **(A)** and 2C6 **(B)**, by anti-HLA-D mAbs: □, no mAb; ○, mAb anti-DQ; △, mAb anti-DP; ▽, mAb anti-DR; ◇, mAb anti-class I used as control. The ascitic fluids were added at a 1:500 dilution.

Fig. 4 shows the results of the inhibition experiments performed with two representative clones. Antigen-specific proliferation of nine of the 14 TLCs tested was inhibited by the addition of anti-DR mAb, and five clones were inhibited by the anti-DQ mAb (Table III). None of them were inhibited by the anti-DP mAb or by an anti-class I mAb used as control. These results indicate that *Parietaria*-specific TLCs can be either HLA-DR– or HLA-DQ–restricted.

TCR Vβ specificity was determined on the same panel of TLCs by means of an indirect fluorescence test performed with 12 mAbs specific for the Vβ segments. The results of this analysis (Table III) indicate that Vβ genes from different families were expressed. A preferential use of specific Vβ families is not evident among the TLCs studied.

Cytokine production by TLCs

IL-2, IL-4, and IFN-γ production was measured on 17 of 40 TLCs after activation with PjE or PMA

TABLE III. Vβ usage and class II restriction of some *Parietaria*-specific TLCs

Exp. 1			Exp. 2		
Clone	Vβ	Restriction	Clone	Vβ	Restriction
1A4	(*)	DR	2A5	2	DQ
1A6	19	DQ	2A8	*	DR
1C3	*	DR	2B2	*	DR
1C10	2	DQ	2C6	3	DQ
1C12	2	DQ	2D4	*	DR
1D3	*	DR	2D6	5.2	DR
1E3	*	DR	2D8	*	DR

Vβ specificity was determined by an indirect fluorescence assay with a panel of mAbs specific for the following Vβ segments: 2, 3, 5.2, 5.3, 8, 11, 13.3, 16, 17, 19, 21, and 22.
*Not recognized by any of the mAbs included in the panel.

plus anti-CD3 mAb. The amount of IL-2 recovered in the culture supernatant was always very low or not detectable with the immunoenzymatic test used, independently of the type of stimulation, and may reflect consumption by receptors on T cells (data not shown). As evident from Table IV, almost all of the TLCs tested were able to produce IL-4 and/or IFN-γ in different amounts, depending on the type of stimulus; PMA plus anti-CD3 mAb produced a greater amount than PjE. PHA-induced activation of the same TLC panel produced results comparable to those obtained after stimulation by PMA plus anti-CD3 mAb (data not shown). Five clones were clearly of T_{H2} phenotype, producing a great amount of IL-4 but not IFN-γ (Table IV). The remaining 12 clones analyzed produced both IL-4 and IFN-γ, thus displaying a T_{H0} phenotype.

The production of IL-4 or IFN-γ by each TLC was scored as positive on the basis of the criteria defined in the Methods section. The results of such analysis are reported in Table V. Most TLCs were able to produce IL-4 after activation by both PjE and PMA plus anti-CD3 mAb (82% and 100%, respectively), whereas a lower number of TLCs was induced to produce IFN-γ in the same conditions (29% and 64%, respectively).

DISCUSSION

The recent advances in the fields of molecular allergology and T-cell regulation of antibody response have suggested new strategies, based on the use of defined peptides administered in a way that results in T-cell inactivation or change in functional properties.[32] Identification and characterization of allergen-specific T cells are thus needed to explore the feasibility of this approach. T-cell clones specific for different allergens (mainly proteins of house dust mites[1, 14-18] Hymenoptera venoms,[19, 20] animal dander,[21] and pollens[2, 22-25]) have been described. Although *P. judaica* is one of the most clinically relevant sources of allergens in the Mediterranean area, to our knowledge, analysis of specific T-cell response at the clonal level has not yet been performed. This study deals with the isolation and characterization of *Parietaria*-specific TLCs.

Our results first indicate that the response to *Parietaria* allergens involves different T cells in the same individual in terms of Vβ usage and HLA restriction. Different results, leading to the conclusion that a limited T-cell repertoire is used in another system that uses house dust mite allergens,[33, 34] have been reported. This discrepancy may reflect a higher degree of complexity of pollen allergens compared with dust mite in which the major allergen Der p 1 accounts for the greatest part of the allergenic activity in the whole extract. Because in the latter case a limited subset of T cells is responsible for the events leading to the disease state, induction of anergy with nonimmunogenic antigens or superantigens can be successfully used.[33, 35] This does not seem to apply to our *Parietaria* model. However, use of multiple peptide vaccines can still be considered, although further studies with isolated, highly purified allergens or characterized epitopes are required to elucidate this point. So far, we have been able to obtain the enrichment of the major allergenic component, Par j 1, by means of hydrophobic chromatography, in the fraction eluate 1 used in this study. However, the complexity of the whole *P. judaica* extract, containing a variety of protein and nonprotein components with or without allergenic activity, makes it difficult to obtain purified components in a suitable amount. This problem can be overcome by alternative approaches based on the recombinant DNA technology. The knowledge of the full amino acid sequence of the relevant protein, obtained by gene sequence analysis,[36] would allow the identification and analysis of the epitopes involved in T- and B-cell activation.

It has been demonstrated that susceptibility to allergic disease is partly determined by products of the major histocompatibility complex. For example, antibody response to highly purified *Ambrosia* allergen, Amb a 5, is strongly associated with expression of HLA-DR2 and Dw2 (DR2.2).[37] These data have been confirmed by analysis of clonal T-cell response.[24] An association between Lol p 3–specific IgE antibody response and HLA-

TABLE IV. Cytokine production by TLCs

Exp. 1					Exp. 2				
Clone		PjE	PMA + α-CD3		Clone		PjE	PMA + α-CD3	
1A4	IL-4	100	>2000	T_{H0}	2A5	IL-4	30	>2000	T_{H0}
	IFN-γ	110	400			IFN-γ	120	340	
1A6	IL-4	15	1700	T_{H0}	2A8	IL-4	200	1550	T_{H2}
	IFN-γ	200	>5000			IFN-γ	<20	40	
1C3	IL-4	93	>2000	T_{H0}	2B2	IL-4	17	1800	T_{H0}
	IFN-γ	140	160			IFN-γ	<20	160	
1C10	IL-4	130	>2000	T_{H0}	2C3	IL-4	1970	ND	T_{H2}
	IFN-γ	180	140			IFN-γ	<20	ND	
1C12	IL-4	150	1900	T_{H0}	2C6	IL-4	<10	195	T_{H0}
	IFN-γ	120	350			IFN-γ	<20	180	
1D3	IL-4	810	ND	T_{H2}	2C11	IL-4	860	>2000	T_{H2}
	IFN-γ	40	ND			IFN-γ	40	50	
1E3	IL-4	185	1740	T_{H0}	2D4	IL-4	75	1750	T_{H0}
	IFN-γ	160	240			IFN-γ	130	210	
1E12	IL-4	710	ND	T_{H2}	2D6	IL-4	340	>2000	T_{H0}
	IFN-γ	<20	ND			IFN-γ	610	>5000	
					2D8	IL-4	540	>2000	T_{H0}
						IFN-γ	100	130	

Cytokine concentrations are expressed in picograms per milliliter. The detection limits of the immunoenzymatic assays used were 10 pg/ml for IL-4 and 20 pg/ml for IFN-γ.
ND, Not done.

DR3 has also been found.[38] In regard to *Parietaria* allergy, high levels of IgE antibody response to the major allergen of *P. officinalis* were positively associated with HLA-DR5.[39] Together with population analysis, contribution of individual major histocompatibility complex class II molecules in T-cell recognition can also be studied to demonstrate HLA association. Studies in this direction have indicated that HLA-DR, as opposed to HLA-DP or HLA-DQ gene products, functions as the major restriction element in the recognition of many allergens.[14, 16] Interestingly, we have found that different clones from the two subjects studied showed both HLA-DR and HLA-DQ molecules as restriction elements for antigen presentation. Although some controversy exists, a functional role of DQ class II molecules in regulating low responsiveness to allergens has been proposed.[40] Indeed, no correlation between HLA-DQ restriction and cytokine profile was found on the TLCs studied, indicating that these clones do not have different functional properties.

Expression of CD40L, a type II membrane protein with homology to tumor necrosis factor-α and tumor necrosis factor-β, on activated T cells plays a central role in regulating IgE synthesis by B cells. In fact, a defect in the CD40L molecule, resulting from either deletions or point mutations within its extracellular domain, has been shown to be responsible for the lack of IgE, IgA, and IgG characteristic of the X-linked immunodeficiency with hyper IgM.[41, 42] Stimulation of B cells through CD40-CD40L interaction induces not only IgE production in the presence of IL-4 or IL-13 but also cell aggregation, proliferation, and CD23 expression.[43] After PMA plus ionomycin induction, all the *P. judaica*–specific TLCs expressed CD40L, indicating that they might be able to interact with B cells for the production of specific IgE. We were not able to detect CD40L on TLCs when activated with PjE and EBV-B cells as antigen presenting cells (F. Sallusto. Unpublished data). This may

TABLE V. Antigen- or mitogen-driven cytokine production by TLCs: Number and percent of positive clones

	PjE		PMA + α-CD3	
	Positive TLCs	Percent	Positive TLCs	Percent
IL-4*	14/17	82	14/14	100
IFN-γ†	5/17	29	9/14	64

*Negativity cutoff for IL-4 was 44 pg/ml.
†Negativity cutoff for IFN-γ was 208 pg/ml.

result from downregulation of CD40L after engagement with CD40 antigen expressed on B cells. Therefore possible differences between T-cell clones in the ability to express CD40L after induction with the specific antigen are not detected by using this approach. One should also consider the possibility that other cell contact–dependent signals may be involved in driving IgE synthesis after T-cell–B-cell interaction, with the expression being different in the different subsets.[44]

Most of the TLCs were able to produce IL-4 after stimulation with PjE or PMA plus anti-CD3 mAb, but antigen induction at the dose tested resulted in a lower frequency of positive clones and in a smaller amount of cytokine produced. Modulation of cytokine profile by the antigen dose has been described in other allergenic systems[19] and could play an important role in classic immunotherapy.

The majority of the *Parietaria*-specific TLCs conformed to the T_{H0}-like subset in that they produced both IL-4 and IFN-γ, although T_{H2}-like TLCs were isolated in the two experiments considered (5 of 17). This finding suggests that a comparable proportion of T_{H0} and T_{H2} populations can be induced in atopic individuals by specific allergen stimulation. Because T_{H1} and T_{H2} may be regarded as memory cells, whereas T_{H0} may represent precursor helper T cells from which T_{H1} and T_{H2} differentiate, the coexistence of T_{H0}- and T_{H2}-like clones in PBMCs of atopic subjects may be explained by different stages of $CD4^+$ T-cell differentiation. Furthermore, the dose and the duration of allergen exposure may influence the T_{H0} or T_{H2} response by atopic individuals. The feature of the *Parietaria* sensitizations (i.e. repeated challenge by inhalation of pollen from this almost ubiquitous and long-pollinating plant) may induce a progressive cycle of T-cell restimulation and consequently support the presence of T cells in different stages of activation and differentiation. Interestingly, the amount of IL-4 produced by both T_{H0}- and T_{H2}-like TLCs was higher after induction by PMA plus anti-CD3 than after induction by PjE, whereas the IFN-γ levels remained comparable. The analysis of the difference in cytokine production, carried out according to the established positivity criteria, indicated that most TLCs were able to produce IL-4 on activation with PjE, whereas a significantly lower number produced IFN-γ in the same conditions (82% and 29%, respectively). The predominant production of IL-4 may be related to a positive regulation of IgE synthesis. Indeed, heterogeneity in terms of amount of IL-4 produced by PBMCs of atopic patients with different sensitizations has been described and related to the levels of IgE produced.[45]

The induction of allergen-specific IgE and activation of different cell types with production of inflammatory mediators are parallel mechanisms involved in the immune response to allergens. Nonetheless, the variability of the T-cell response against different allergenic substances highlights the importance of further exploration of the mechanism involved. Therefore, *Parietaria*-specific TLCs could represent a useful tool for the evaluation of in vitro models, leading to improvement in therapeutic approaches directed at silencing the T cells involved in allergic disorders.

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