Major Histocompatibility Complex–independent Recognition of a Distinctive Pollen Antigen, Most Likely a Carbohydrate, by Human CD8$^+$ α/β T Cells

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Summary

We have isolated CD8$^+$ α/β T cells from the blood of atopic and healthy individuals which recognize a nonpeptide antigen present in an allergenic extract from *Parietaria judaica* pollen. This antigen appears to be a carbohydrate because it is resistant to proteinase K and alkaline digestion, is hydrophilic, and is sensitive to trifluoromethane-sulphonic and periodic acids. In addition, on a reverse-phase high performance liquid chromatography column the antigen recognized by CD8$^+$ T cells separates in a fraction which contains >80% hexoses (glucose and galactose) and undetectable amounts of proteins. Presentation of this putative carbohydrate antigen (Pj^{CHO}Ag) to CD8$^+$ T cell clones is dependent on live antigen presenting cells (APCs) pulsed for >1 h at 37°C, suggesting that the antigen has to be internalized and possibly processed. Indeed, fixed APCs or APCs pulsed at 15°C were both unable to induce T cell response. Remarkably, Pj^{CHO}Ag presentation is independent of the expression of classical major histocompatibility complex (MHC) molecules or CD1. CD8$^+$ T cells stimulated by Pj^{CHO}Ag-pulsed APCs undergo a sustained $[Ca^{2+}]_i$ increase and downregulate their T cell antigen receptors (TCRs) in an antigen dose– and time-dependent fashion, similar to T cells stimulated by conventional ligands. Analysis of TCR Vβ transcripts shows that six independent Pj^{CHO}Ag-specific T cell clones carry the Vβ8 segment with a conserved motif in the CDR3 region, indicating a structural requirement for recognition of this antigen. Finally, after activation, the CD8$^+$ clones from the atopic patient express CD40L and produce high levels of interleukins 4 and 5, suggesting that the clones may have undergone a Th2-like polarization in vivo. These results reveal a new class of antigens which triggers T cells in an MHC-independent way, and these antigens appear to be carbohydrates. We suggest that this type of antigen may play a role in the immune response in vivo.

It is well known that T lymphocytes expressing α/β TCR recognize antigenic peptide bound to MHC molecules. However, there is increasing evidence that nonprotein antigens can also be recognized by T cells (for review see reference 1). A first category of nonprotein antigens is represented by lipids or glycolipids such as mycolic acid and lipoarabinomannan (2–4). Presentation of these antigens to α/β T cells requires processing and is carried out by CD1 molecules which display a specificity for hydrophobic antigens (1). A second class of nonprotein antigens includes low mol wt phosphorylated molecules such as isopentenyl pyrophosphate and its synthetic analogs (5–7). In this case presentation to γ/δ T cells is independent from uptake and processing and does not require any known antigen presenting molecule.

A long-standing question is whether carbohydrates may be recognized by T cells. It has been shown that MHC molecules can bind glycopeptides via the peptide moiety and display the carbohydrate for interaction with TCR (8–11). However, this type of recognition still can be regarded as a variation of conventional peptide antigen recognition as it has been shown for haptenized peptide (12). So far, there has been neither evidence that carbohydrates may bind directly to MHC or other antigen presenting molecules (13, 14) nor reports that carbohydrates as such might trigger T cells in the absence of MHC molecules.

We have observed that T cells which proliferate in vitro

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in response to a pollen extract from *Parietaria judaica* (PjE)[1], a common environmental allergen of the Mediterranean area (15), comprise a sizable fraction of $CD8^+$ α/β T cells. Here we provide evidence that these T cells recognize a putative carbohydrate antigen present in PjE. Presentation of Pj^{CHO}Ag to T cells is dependent on APCs, but is not restricted by either MHC or CD1 molecules. These results reveal a new mode of antigen presentation and a new class of ligands which can trigger α/β T cells.

Materials and Methods

PjE Reactive T Cell Clones. PBMCs from one atopic and one healthy individual were cultured in 96-well flat-bottomed plates (Costar Corp., Cambridge, MA) in RPMI 1640 supplemented with 2 mM l-glutamine, 1% nonessential amino acids, 1% pyruvate, 50 μg/ml kanamycin, 5×10^{-5} M 2-ME (GIBCO BRL, Gaithersburg, MD) and 5% human serum (Sigma Chemical Co., St. Louis, MO) in the presence of PjE (50 μg protein/ml). Recombinant IL-2 (20 U/ml; provided by Dr. A. Lanzavecchia, Basel Institute for Immunology, Basel, Switzerland) was added at day 6. After 20 d, T cell blasts were cloned by limiting dilution. T cell clones were maintained by periodic restimulation with phytohemagglutinin, irradiated allogeneic PBMCs (3,000 rad from a ^{60}Co source), and rIL-2 as previously described (16).

Cell Lines. Dendritic cells (DCs) were generated by culturing peripheral blood monocytes in the presence of GM-CSF and IL-4 (17). T2 (TAP-deficient), and 221 (class I–deficient) were provided by Dr. A. Lanzavecchia. SJO (class II–deficient) was a gift of Dr. J. Gorsky (Blood Research Institute, Milwaukee, WI). C1R (class I–deficient) stably transfected with the CD1a, CD1b, or CD1c genes or mock transfected were provided by Dr. M. Brenner (Brigham and Women's Hospital, Boston, MA). EBV-transformed lymphoblastoid cell lines (B-LCL) were derived from PBMCs as described (18). All cell lines were maintained in 10% FCS-RPMI (Hyclone Labs., Inc., Logan, UT).

FACS® Analysis. Cell staining was performed using mouse mAbs followed by FITC- or PE-conjugated affinity-purified, isotype specific goat anti–mouse antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL). The following antibodies were used: OKT8 (IgG2a, anti-CD8; American Tissue Culture Collection, Rockville, MD), 6D10 (IgG1, anti-CD4; provided by Dr. E. Roosnek, University of Geneva, Geneva, Switzerland); PE-Leu2a (IgG1, anti-CD8; Becton Dickinson, Mountain View, CA); FITC–anti-TCR α/β (IgG1; Becton Dickinson); B1 (IgG1, anti–pan-γ/δ, provided by Dr. G. De Libero, University of Basel, Basel, Switzerland); a panel of mAbs recognizing different Vβ gene products (Immunotech, Marseille, France); and TRAP-1 (IgG1, anti-hCD40L; provided by Dr. R. Kroczek, University of Berlin, Berlin, Germany). The samples were analyzed on a FACScan® using propidium iodide to exclude dead cells.

Antigen Presentation Assay. T cells were cultured with APC in the presence of various concentrations of antigens in 200 μl human serum–RPMI in flat-bottomed microplates. Cultures were pulsed with 1 μCi of [^{3}H]thymidine (2 Ci/mmol; Amersham International, Little Chalfont, UK) on day 2 and harvested 16 h later. For antibody blocking studies, cultures were carried out in the presence of different antibodies provided as 1:10 dilution of culture supernatant. The following mAbs were used: W6/32 (IgG2a, anti–HLA class I), L243 (IgG2a, anti–HLA-DR), SPVL3 (IgG2a, anti–HLA-DQ), and B7/21 (IgG1, anti–HLA-DP). APCs were either irradiated or treated with 1 mg/ml mitomycin C (Sigma Chemical Co.) for 30 min at 37°C. In some experiments the APC were fixed with 0.05% glutaraldehyde for 30 s and blocked with 0.1 M lysine in PBS for 5 min before or after antigen pulse.

Cytokine Production. T cells were stimulated for 40 h with 10^{-7} M PMA (Sigma Chemical Co.) and anti-CD3 antibody (TR66, 0.2 μg/ml). Cytokine production was quantified in the supernatants by ELISA (PharMingen, San Diego, CA; reference 19).

Preparation of PjE and Removal of Proteins, Phosphates, and Carbohydrates. PjE was prepared by extracting PjE pollen (Allergon, Angelholm, Sweden) with 0.125 M bicarbonate buffer as described (20). The extract contained ~20% protein as assessed by the Bradford method (21). PjE was also purchased from Neo-Abello (Madrid, Spain). To remove proteins from the extract two methods were used. First, 5 mg PjE was digested in 200 μl PBS, pH 7.2, with 100 μg proteinase K (PK; Sigma Chemical Co.) in the presence of 1 mM $CaCl_2$ for 2 h at 37°C followed by heating at 65°C for 5 min. The digestion was repeated twice. Alternatively, 5 mg PjE was incubated in 100 μl 3 M NaOH/0.1 M $NaBH_4$ overnight at room temperature. The samples (referred to as ^{PK}PjE and ^{NaOH}PjE, respectively) were tested and compared to the same concentration of untreated or mock-treated PjE. To remove phosphates, PjE (2.5 mg) was treated with 10 U phosphatase A (Sigma Chemical Co.) in 100 μl of 50 μM Tris/HCl, pH 8.3, for 2 h at 37°C. The enzyme was inactivated by heating to 65°C for 10 min. To remove sugars two methods were used. PjE (10 mg) was treated for 1 h at 0°C with trifluoromethane-sulphonic acid (TFMS) and anisole (1:2 ratio) as described (22). After incubation, an equal volume of ice-cold aqueous pyridine (60% vol/vol) was added. Alternatively, PjE (5 mg/ml) was incubated in 10 mM acetate buffer, pH 4.7, containing 10 mM sodium periodate (SP; Sigma Chemical Co.) for 6 h at 4°C in the dark (23). The reaction was stopped with a molar excess of ethylene glycol and $NaBH_4$ was added at 2 mg/ml. The samples (referred to as ^{TFMS}PjE and ^{SP}PjE, respectively) were tested and compared to the same concentration of untreated or mock-treated PjE.

Purification of the Antigen Recognized by $CD8^+$ T Cell Clones. The antigen recognized by $CD8^+$ T cell clones was purified from crude pollen extract using two different protocols. In the first protocol, PjE was dissolved in 4% trichloroacetic acid (15 mg/ml) and precipitated proteins were removed by centrifugation. The supernatant was neutralized by 1 N NaOH and lyophilized. The sample was fractionated on a preparative reverse-phase column (RP-8; Waters, Millipore Corporation, Bedford, MA) by eluting with a linear gradient of methanol in water. Collected fractions were concentrated at reduced pressure, lyophilized, and tested for biological activity as well as for carbohydrate and protein content. In the second protocol, PjE (300 mg in 80 ml water) was extracted (80:65, vol/vol aqueous to organic) with chloroform/methanol (2:1, vol/vol) and the two phases were separated by centrifugation. The upper layer and the interface were recovered and lyophilized, and the lower layer was dried under nitrogen. Most of the biological activity was found in the upper fraction. The water soluble material (92 mg) was dissolved in 12 ml water and separated using two cycles of centrifugal concentrators with a cutoff of 10 and 3 kD (Amicon Inc., Beverly, MA). The biologi-

[1] *Abbreviations used in this paper:* B-LCL, EBV-transformed lymphoblastoid cell lines; DC, dendritic cell; GC-MS, gas chromatography–mass spectrometry; PjCHOAg, carbohydrate antigen present in PjE; PjE, *Parietaria judaica* pollen extract; rpHPLC, reverse-phase HPLC; SP, sodium periodate; TFMS, trifluoromethane-sulphonic acid.

cal activity was recovered in the filtrate of the 10-kD concentrator and was only partially retained in the 3-kD concentrator. The 3-kD filtrate was lyophilized to give 11 mg residue. This was further fractionated on a reverse phase HPLC (rpHPLC; RP-18; Waters) by eluting with a gradient of methanol in water. The fractions were tested for biological activity as well as for carbohydrate and protein content. The sugar content in the chromatographic fractions was measured according to Ashwell (24). Briefly, 0.1 mg of the lyophilizate was dissolved in 2 ml water and treated with 0.05 ml phenol reagent (80% by weight of redistilled phenol in water) and 5 ml of concentrated sulfuric acid. The sample was kept at room temperature for 30 min and the optical density was determined at 490 nm. The amount of carbohydrate content was estimated by comparison to a standard curve of glucose. The protein content was determined by a protein assay (Bio-Rad, Hercules, CA) using BSA as standard.

Sugar Analysis on Active Antigenic Fractions. Samples from the active chromatographic fractions were hydrolyzed with 2 M TFA followed by reduction with $NaBH_4$ to give a mixture of alditols. Such compounds were acetylated by acetic anhydride and pyridine to yield a mixture of alditol acetates, which were analyzed by gas chromatography–mass spectrometry (GC-MS). To exclude the interference of free monosaccharides, a mock control was performed after acetylation of an untreated aliquot of the samples.

$[Ca^{2+}]_i$ Measurement. T cell clones were loaded with Indo-1 AM (Sigma Chemical Co.) as described (25). Cells were mixed at a 1:2 ratio with autologous B-LCL cells which had been pulsed with PjE or ^{NaOH}PjE. The cells were centrifuged at 1,500 rpm for 1 min at 4°C to allow conjugate formation, heated for 1 min at 37°C, and analyzed on a Coulter Elite Flow Cytometer (Coulter Electronics Inc., Hialeah, FL) to detect Ca^{2+} fluxes in conjugated T cells (25). Only live (based on forward and side scatter criteria) and Indo-1–loaded cells were included in the analysis. As positive control we recorded Ca^{2+} fluxes in T cells after cross-linking of CD3 with a specific mAb (clone TR66).

V β8 TCR-CDR3 Sequencing. Three clones from the atopic patient and three clones from the normal control were further analyzed by CDR3 spectratyping as previously described (26). Once it was proved that the clones contained only one genomic sequence, direct sequencing was performed. The cDNA obtained from the relevant clones was amplified for the Vβ8 TCR family according to a published procedure (27). The Vβ8-specific PCR products were purified by means of a Microspin G50 column (Pharmacia Biotech, Uppsala, Sweden) to eliminate unincorporated primers. After purification 0.5-μl PCR products were used for cycle sequencing reaction according to instructions (Perkin-Elmer, Pasadena, CA) using sense and antisense internal primers in different PCR reactions. After amplification the samples were dried, boiled for 2 min in 4 μl loading dye, and then loaded on a fluorescence based sequencer (ABI373, Perkin-Elmer) using a denaturing 40% acrylamide gel. The samples were run for 8 h and the data were collected and analyzed using a 373 sequencing software for base calling.

Results

Isolation of $CD8^+$ T Cell Clones Specific for PjE. PBMCs from a PjE-sensitive patient and one nonatopic individual were stimulated in vitro with PjE. The responding cells were expanded with IL-2. After 20 d the cell lines were enriched for antigen-specific T cells as shown by a strong proliferative response to the inducing agent in the presence of autologous mononuclear cells (data not shown). Interestingly, in several independent cell lines from both individuals a sizable fraction of the T cells (20–30%) was $CD8^+$ (Fig. 1 *A*). Since it is unusual to find $CD8^+$ T cells in cultures stimulated by protein antigens, we isolated a number of $CD4^+$ and $CD8^+$ α/β T cell clones (Fig. 1 *B*) by limiting dilution and tested their capacity to recognize PjE.

As shown in Table 1, several $CD8^+$ T cell clones isolated from the atopic patient and the healthy individual proliferated in response to PjE. Proliferation was specific since it was observed using different sources of PjE, while pollen extracts from unrelated species such as *Cupressus arizonica* and *Olea europaea* were ineffective (not shown). Interestingly, while $CD8^+$ clones from the healthy control produced only IFN-γ, those isolated from the allergic patient produced high levels of IL-4 and IL-5 (Table 1) and expressed CD40L after stimulation (Fig. 1 *C*), suggesting that they may have undergone a Th2-like polarization in vivo.

α/β^+ $CD8^+$ T Cell Clones Recognize a Proteinase K–resistant, Phosphatase-resistant, Periodate-sensitive Antigen. The nature of the antigen recognized by $CD8^+$ PjE-specific T cell clones was investigated. The crude pollen extract was digested with proteinase K or subjected to alkaline hydroly-

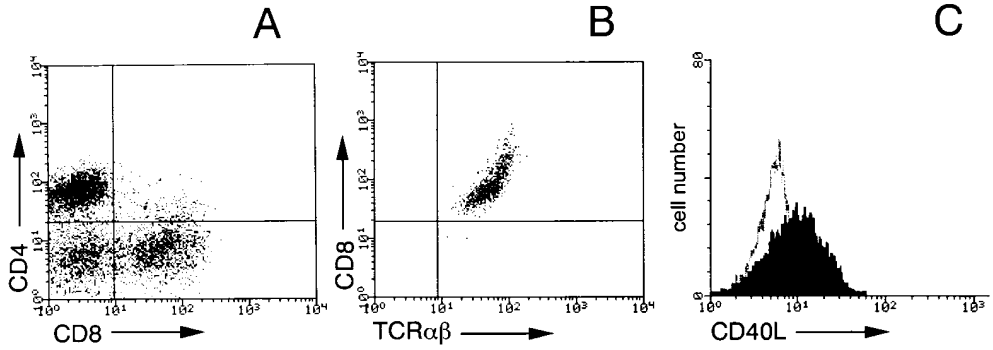

Figure 1. (*A*) CD4 and CD8 expression in a short-term PjE-reactive T cell line from atopic donor. PBMCs were stimulated in vitro with PjE. After 20 d T cell blasts were stained with anti-CD8 and anti-CD4 mAbs. (*B*) PjE-specific $CD8^+$ T cell clones express α/β TCR. Clone P4.2 was stained with PE-labeled anti-CD8 and FITC-labeled TCR-α/β mAbs. (*C*) Expression of CD40L by $CD8^+$ T cell clones. Clone P4.2 was activated with PMA + ionomycin for 8 h and stained with anti-CD40L mAb. Stimulated cells (*filled histogram*) were compared with unstimulated cells (*open histogram*).

Table 1. *Proliferative Response and Cytokine Profile of PjE-specific CD8⁺ T Cell Clones from Atopic (A) and Nonatopic (B) Individuals*

	^{3}H-TdR	IL-4	IL-5	IFN-γ		^{3}H-TdR	IL-4	IL-5	IFN-γ
	cpm × 10⁻³	*pg/ml*	*pg/ml*	*pg/ml*		*cpm × 10⁻³*	*pg/ml*	*pg/ml*	*pg/ml*
Donor A					Donor B				
P4.2	30.5*	537‡	746‡	>4,000*	CP.27	23.4	<20	<20	2,395
P4.4	23.5	<20	<20	>4,000	CP.28	18.7	35	<20	>4,000
P4.6	33.9	222	959	>4,000	CP.31	14.6	<20	<20	3,000
P4.15	27.3	356	<20	>4,000	CP.38	25.6	<20	<20	2,610
P4.16	23.9	232	<20	2,821	CP.44	10.7	ND	ND	ND
P4.17	42.3	80	543	>4,000	CP.45	7.4	ND	ND	ND
P4.26	44.3	1,378	1,300	3,394	CP.53	26.0	ND	ND	ND
P4.27	29.0	664	319	>4,000	CP.56	10.0	ND	ND	ND
P4.33	30.3	518	>2,000	2,624	CP.66	27.8	<20	<20	>4,000
P4.52	36.1	162	<20	3,359	CP.68	69.2	102	<20	>4,000

*T cells were cultured with PjE (50 μg/ml) and autologous B-LCL. Proliferative response was measured on day 3. Background response in the absence of antigen for all clones was $<10^{-3}$.
‡T cells were stimulated with 10^{-7} M PMA + 0.2 μg/ml mAb TR66 (anti-CD3). After 40 h supernatants were collected and tested for cytokines by ELISA.

sis. Both treatments destroyed proteins and abolished stimulation of PjE-specific CD4⁺ T cells, but did not affect and actually enhanced stimulation of the CD8⁺ clones, suggesting that CD8⁺ T cells recognize a nonprotein antigen (Fig. 2). Treatment with alkaline phosphatase did not decrease the capacity of PjE to stimulate CD8⁺ clones, indicating that the antigen does not contain critical phosphate residues (data not shown). When PjE was extracted with chloroform/methanol, the antigen recognized by CD8⁺ T cells separated in the aqueous phase (see below). Taken together, these properties suggested that the antigen recognized might be a carbohydrate. Therefore, we tested the sensitivity of the antigen to treatments which destroy sugars. In contrast to protease and phosphatase treatments, both periodic acid and TFMS treatment of PjE completely abolished recognition by CD8⁺ T cell clones, while the capacity to stimulate CD4⁺ T cell clones was not affected (Fig. 3). These results suggest that the antigen recognized by CD8⁺ T cell clones is not affected by removal of proteins or phosphate groups but is sensitive to treatment which degrades carbohydrates or related polyhydrate compounds.

Partial Purification of the Antigen Recognized by CD8⁺ T Cell Clones. In a first attempt to purify the antigen recognized by CD8⁺ T cell clones, we separated the material remaining after protein precipitation by a semipreparative reverse-phase column. The activity was recovered in several fractions and the most active was enriched in carbohydrates (83%) but still contained ~1% proteins (data not shown). In a second attempt, we combined phase fractionation with ultrafiltration and rpHPLC. After extraction with chloroform/methanol, the antigen recognized by CD8⁺ clones separated in the aqueous phase, which contains mainly polar molecules, but was absent at the interface and in the or-

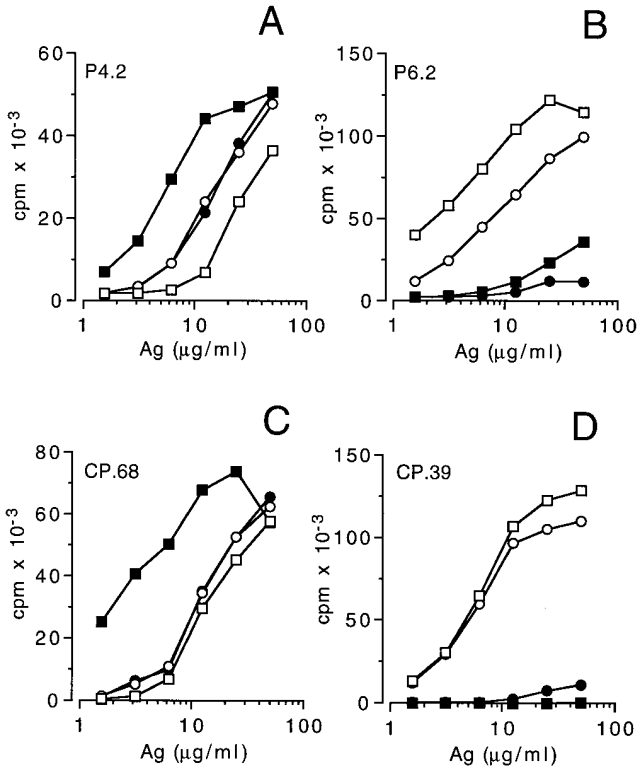

Figure 2. CD8⁺ T cell clones recognize a proteinase K (PK)–resistant, alkaline-resistant antigen of PjE. CD8⁺ T cell clones P4.2 (*A*) and CP.68 (*C*) and CD4⁺ T cell clones P6.2 (*B*) and CP.39 (*D*) were stimulated with autologous irradiated B-LCL in the presence of different concentration of PjE (□), ^{PK}PjE (●), or ^{NaOH}PjE (■). A mock control for proteinase K (○) was also included. Proliferative response was measured on day 3. Digestion with pronase gave comparable results (not shown).

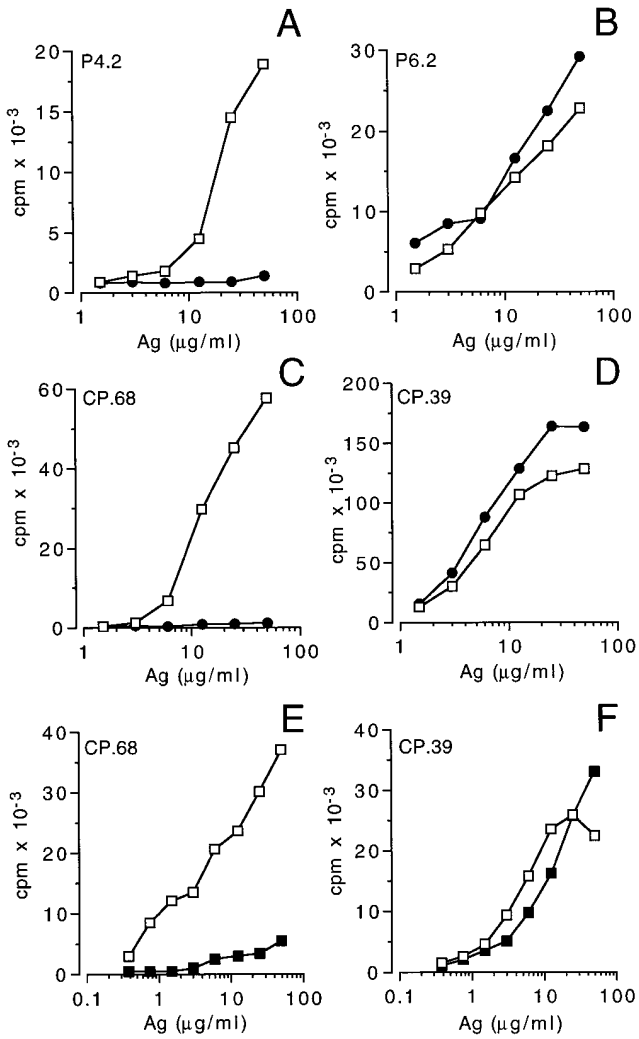

Figure 3. Oxidation with periodic acid and treatment with TFMS completely abolished recognition of PjE by $CD8^+$ T cell clones. $CD8^+$ T cell clones P4.2 (*A*) and CP.68 (*C* and *E*) and $CD4^+$ T cell clones P6.2 (*B*) and CP.39 (*D* and *F*) were cultured with autologous irradiated B-LCL in the presence of different concentration of PjE (□), ^{TFMS}PjE (●), or ^{SP}PjE (■). Proliferative responses were measured on day 3. A mock control without periodate gave comparable results as PjE (data not shown).

ganic phase (data not shown). The hydrophilic fraction was separated using centrifugal concentrators with cutoff of 10 and 3 kD. The biological activity was recovered in the filtrate of the 10-kD concentrator and was partially retained in the 3-kD concentrator. The 3-kD filtrate was further fractionated on an rpHPLC by elution with a gradient of methanol in water (Fig. 4 *A*). As expected, the material with biological activity eluted very early while most of the contaminating compounds still present in the 3-kD filtrate were retained by the column and were eluted at later time points (Fig. 4 *B* and data not shown). The positive fraction was ~20-fold more active on a weight basis than the starting material (Fig. 4 *B*, *inset*). When this fraction was analyzed by GC-MS, the chromatographic elution and the mass spectrum revealed the presence of glucose and galactose at a molar ratio of 47:53 (Fig. 4 *C*). Furthermore, the same fraction contained no detectable peptides as assayed by the Bradford method (17), direct and reverse-phase TLC of the hydrolyzed fraction (HCl 6 N, 110°C, 6 h) followed by ninidrin staining, Edman degradation, and amino acid analysis using a 494 protein sequencer (data not shown). To further exclude any peptide contamination, the active fraction was digested with carboxypeptidase Y, a protease which digests most peptides including those with blocked NH_2 termini (27). This treatment did not affect the bioactivity of the purified antigen but completely abolished the ability of a tetanus toxoid peptide to stimulate a specific $CD4^+$ T cell clone (data not shown). Taken together the above results suggest that, according to two different criteria (i.e., sensitivity to chemical treatment and purification with two methods), the antigen recognized by $CD8^+$ clones is a low mol wt polyhydrate molecule, possibly a carbohydrate.

APC-dependent and MHC- and CD1-independent Presentation of Pj^{CHO}Ag to $CD8^+$ T Cell Clones. The requirements for antigen recognition were studied using as readout T cell proliferation and cytokine production. The $CD8^+$ clones proliferated in response to PjE only in the presence of APC (Fig. 5 *A*). Optimal presentation was provided by B-LCL and PBMCs, while, surprisingly, DCs were considerably less efficient (Fig. 5, *A*, and *B*). Several unrelated B-LCL tested were able to function as APCs for the $CD8^+$ clones (Fig. 5 *A*), even if they lacked class I (221 and C1R) or class II molecules (SJO and T2) (Fig. 5 *C*). In addition, antibodies to class I and class II molecules did not inhibit T cell activation (Fig. 5 *D*), while they did inhibit antigen recognition by class I– and class II–restricted T cell clones (data not shown). A contribution of CD1 molecules was also ruled out by the finding that (*a*) anti-CD1 antibodies did not affect the proliferative response and (*b*) CD1-a, -b, and -c transfectants were as efficient as the untransfected control (data not shown). Taken together, these data suggest that none of the known antigen-presenting molecules are involved in the presentation of Pj^{CHO}Ag to $CD8^+$ α/β T cell clones.

The requirements for antigen uptake and processing were studied using $[Ca^{2+}]_i$ increase as a sensitive and rapid assay of T cell antigen recognition. No response was observed when the $CD8^+$ T cells were incubated with the antigen in solution, confirming the requirements for APCs (Fig. 6 *D*). In contrast, the clones showed a strong and sustained $[Ca^{2+}]_i$ increase when conjugated with PjE-pulsed APC. Indeed, a time course of antigen pulsing revealed that a short incubation with antigen (1 h) was not sufficient for presentation (Fig. 6 *B*) and that the stimulatory capacity was progressively acquired during the next 5 h of incubation (Fig. 6 *C*). While APC fixed after antigen pulse retained the ability to induce $[Ca^{2+}]_i$ increase in T cell clones (data not shown), APC fixed before a 5-h pulse at 37°C with PjE were ineffective (Fig. 6 *E*). In addition, live APC pulsed at 15°C failed to stimulate T cells (Fig. 6 *F*). Interestingly, chloroquine did not inhibit presentation of Pj^{CHO}Ag (data not shown), suggesting that processing in an acidic

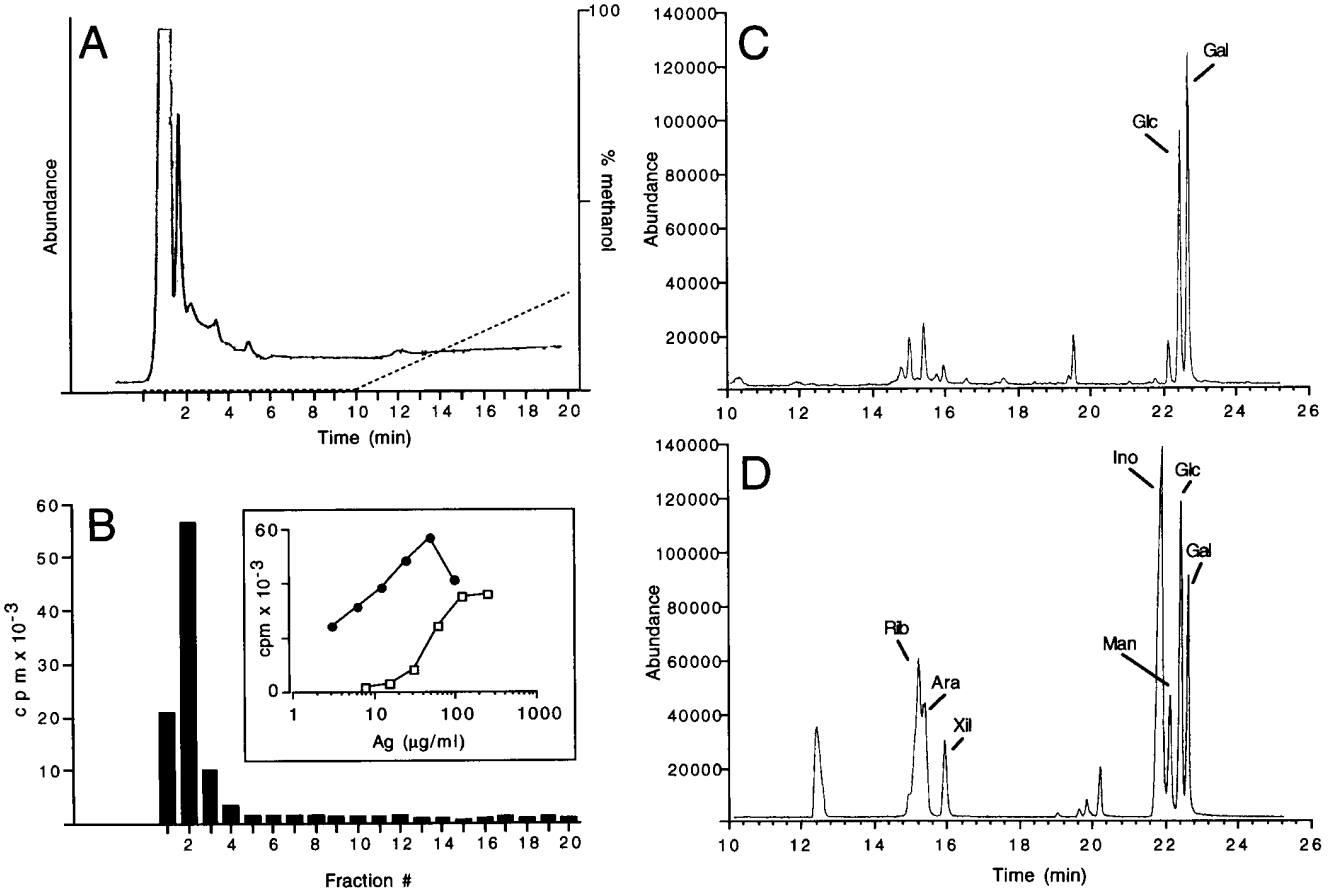

Figure 4. Purification of the antigen recognized by $CD8^+$ T cell clones by rpHPLC. (*A*) The sample recovered after phase fractionation and ultrafiltration was separated on an rpHPLC by eluting with a linear gradient of methanol in water starting at fraction 10 (flow 0.8 ml/min). 1-ml fractions were collected and tested for activity in T cell proliferation assay. Shown is the first 20-min profile; the dotted line represents the methanol gradient. Note that most of the material retained on the column eluted at later time points which are not shown. (*B*) Proliferative response of clone P4.26 to rpHPLC fractions in the presence of autologous B-LCL. Fractions 21–60 were inactive (not shown). The inset shows the titration of fraction 2 (●) compared to the starting material PjE (□) in proliferation assay. (*C* and *D*) Sugar composition of fraction 2 (*C*) was determined by GC-MS and compared to a standard mixtures of sugars (*D*). *Glc*, glucose; *Gal*, galactose.

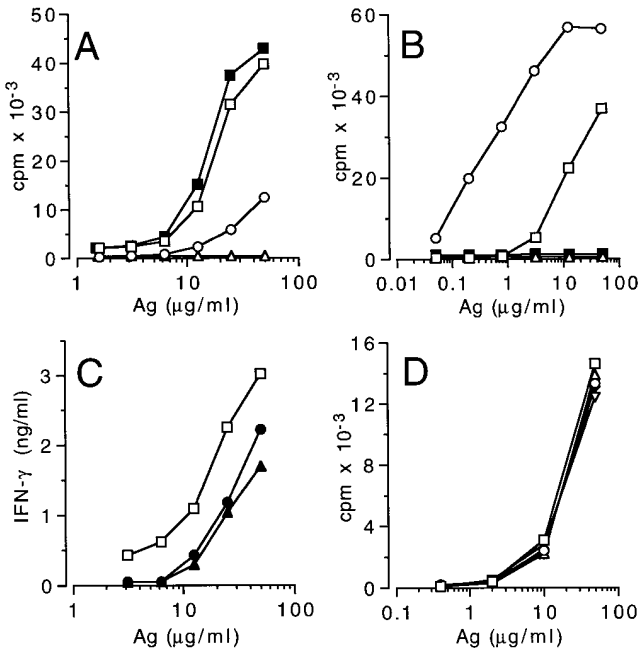

Figure 5. $Pj^{CHO}Ag$ is more efficiently presented to $CD8^+$ T cells clone by B-LCL than DCs and its presentation does not require MHC class I or class II molecules. Proliferative response of $CD8^+$ T cell clone P4.2 (*A*) and $CD4^+$ T cell clone P6.2 (*B*) to different concentrations of PjE in the absence of APC (△) or in the presence of autologous B-LCL (□), MHC-unrelated B-LCL (■), or DC (○). (*C*) IFN-γ production by the $CD8^+$ T cell clone P4.2 in response to PjE in the presence of autologous B-LCL (□), T2 (▲), or 221 (●) cells. Similar results were obtained using C1R and SJO cells, which lack class I and class II molecules, respectively. (*D*) Proliferative response of $CD8^+$ T cell clone P4.2 to PjE in the absence (□) or in the presence of mAbs to HLA-class I (△), HLA-DR (▽), HLA-DQ (○), or HLA-DP (▽) molecules.

compartment was not involved. Finally, APC retained the capacity to stimulate T cells after removal of the antigen (Fig. 6, *G–I*). By comparing the stimulatory capacity of APC chased for different times with that of APC pulsed with different concentrations of antigen, we estimated the half-life of antigen on pulsed APC to be ~1 h. Taken together these results suggest that $Pj^{CHO}Ag$ has to be captured by the APC and it is displayed on their surface in a stable

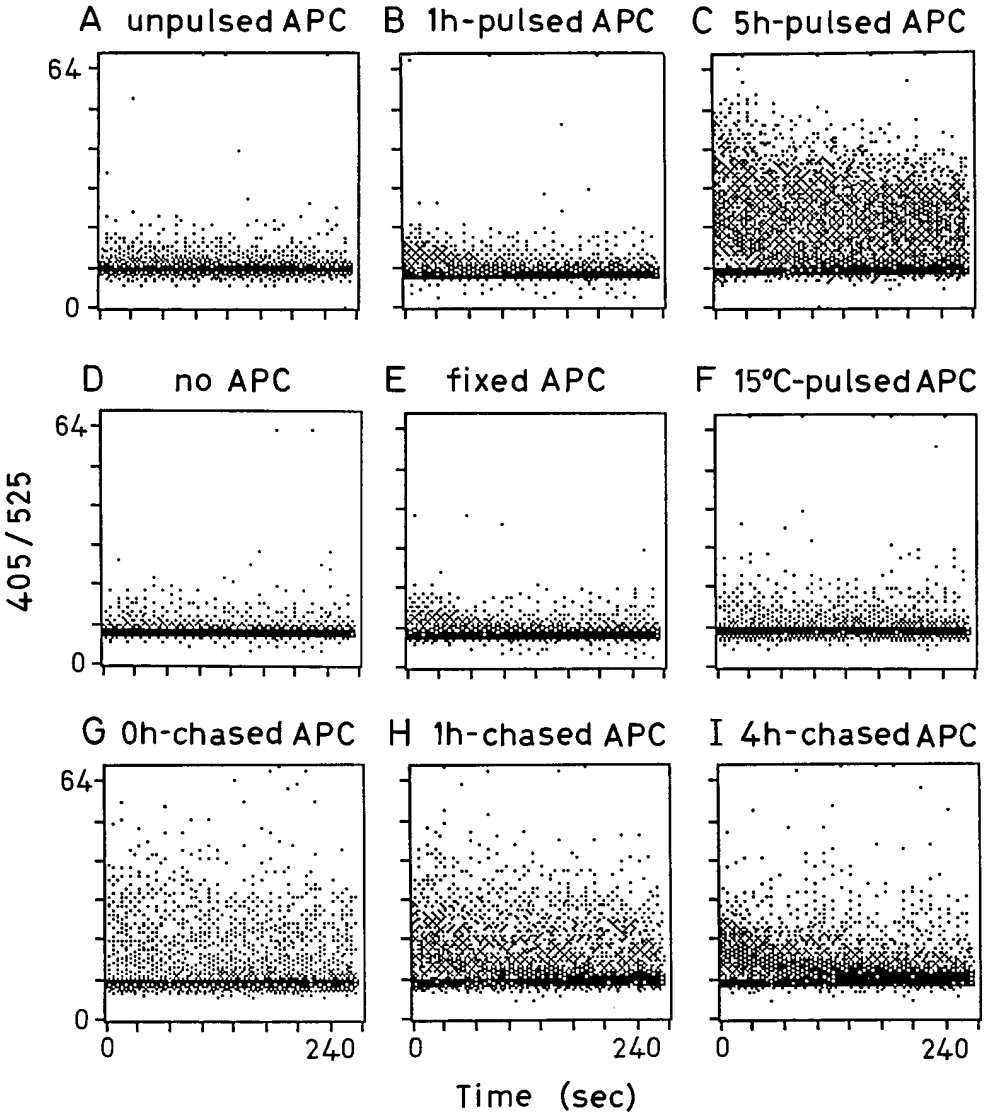

Figure 6. Conditions required for pulsing APC with $Pj^{CHO}Ag$. $[Ca^{2+}]_i$ in T cells (clone P4.2) was measured in the presence of: (*A*) unpulsed APC; (*B* and *C*) APC pulsed at 37°C with ^{NaOH}PjE for 1 h (*B*), or 5 h (*C*); (*D*) ^{NaOH}PjE in solution; (*E*) fixed APC pulsed at 37°C with ^{NaOH}PjE for 5 h; (*F*) APC pulsed at 15°C with ^{NaOH}PjE for 5 h; (*G–I*) APC pulsed overnight with ^{NaOH}PjE, washed, and chased for 0 (*G*), 1 (*H*), or 4 (*I*) h. Comparable results were obtained using PjE as antigen. In a separate experiment we found that the magnitude of $[Ca^{2+}]_i$ increase induced by PjE is comparable to that induced by cross-linked anti-CD3 (data not shown).

fashion, possibly associated with molecules preferentially expressed on B cells.

TCR-β Usage and Evidence for the Involvement of TCR in the Recognition of $Pj^{CHO}Ag$. All $CD8^+$ $Pj^{CHO}Ag$-specific T cell clones isolated from two individuals express the Vβ8 chain as detected by mAbs. This finding is compatible with stimulation by a Vβ8-specific superantigen possibly present in the allergenic extract. However, this possibility was ruled out by the finding that polyclonal $V\beta8^+$ lines were not stimulated by PjE (data not shown). Since we could exclude a superantigen type of recognition we searched for a conserved motif in the CDR3 region (Table 2). Sequencing of the Vβ chains of six independent T cell clones revealed a remarkable conservation in positions 3 and 5 of the CDR3 region (threonine and valine, respectively), while the length differed by only one amino acid. In addition, all six clones analyzed used the Jβ1.2 segment. We also investigated the kinetics of T cell activation by $Pj^{CHO}Ag$. As shown in Fig. 7, $CD8^+$ T cells exposed to PjE-pulsed APC undergo a $[Ca^{2+}]_i$ increase that is sustained for >30 min (Fig. 7 *A*) and downregulate their TCRs in an antigen dose– and time-dependent fashion (Fig. 7 *B* and data not shown). These results demonstrate that $Pj^{CHO}Ag$ triggers the TCR with the same kinetics as conventional TCR ligands such as peptide–MHC complexes.

Discussion

We have shown that $CD8^+$ α/β T cells can specifically recognize a carbohydrate antigen present in a pollen extract. This antigen differs from other nonpeptide antigens (2–7, 28) because of its chemical nature and the lack of MHC or CD1 restriction.

Several lines of evidence suggest that the stimulatory ligand is a carbohydrate. First, it is resistant to treatments that destroy proteins, such as proteinase K digestion and alkaline treatment, excluding a possible contribution of a peptide. Second, it separates in the aqueous phase, ruling out the possibility that it may contain lipids. Third, it is sensitive to agents that destroy polysaccharides, such as

Table 2. *Nucleotide and Predicted Amino Acid Sequences of TCR-β Chains from Pj^{CHO} Ag-specific CD8$^+$ T Cell Clones from Two Different Donors*

Clone	Vβ	CDR3	Jβ
Donor A			
P4.2	8	C A S S P G **T** G **V** D G Y T F tgtgccagtagtcct<u>gggacaggggg</u>tggatggctacaccttc Dβ1	1.2
P4.17	8	C A S S L V **T** G **V** T D G Y T F tgtgccagtagtttggtaacaggcgtgacagatggctacaccttc	1.2
P4.52	8	C A S S Q A **T** A **V** N G Y T F tgtgccagtagtcaagccacagcggtgaacggctacaccttc	1.2
Donor B			
CP.31	8	C A S S V A **T** A **V** T C G Y T F tgtgccagtagtgtcgccacagcggtgacatgcgcctacacgttc	1.2
CP.38	8	C A S S G **T** S **V** A M G Y T F tgtgccagtagt<u>gggacag</u>atgtggccatgggttacaccttc Dβ1	1.2
CP.68	8	C A S S V A **T** A **V** T D G Y T F tgtgccagtagtgtcgccacagcggtgacagatggctacaccttc	1.2

CDR3 regions run from the consensus cysteine to the first phenylanaline of the "FGXT" motif found in the J region. Identity of Dβ segments was assigned when at least six consecutive bases identical to those of a germline Dβ segment were identified within the junctional region.

TFMS and periodic acid. Finally, it is enriched in a fraction containing high levels of glucose and galactose. However, we cannot exclude that other polyhydrate molecules with similar sensitivity to chemical treatments may be responsible for the stimulation of Pj^{CHO}Ag-specific T cell clones. Although the structure of the epitope still has to be defined, it is likely to be intracellularly generated since we have found that, in order to stimulate specific T cells, Pj^{CHO}Ag has to be pulsed with live APC for >1 h. Indeed, fixed APC or APC pulsed at 15°C were both unable to induce T cell response. We have found that chloroquine does not interfere with presentation of Pj^{CHO}Ag, suggesting that either processing of this antigen is not required or may involve a different intracellular compartment. More evidence is needed to elucidate these points.

A remarkable finding is that recognition of Pj^{CHO}Ag, unlike recognition of other ligands by α/β T cells, does not require the expression of classical MHC class I, class II, and CD1 molecules. However, once generated the epitope remains associated with the cell surface with a half life of ~1 h. We envisage two possibilities to explain how this epitope is displayed on APC surface for T cell recognition. First, the antigen may form a complex with a nonpolymorphic molecule which functions as classical antigen-presenting molecules, i.e., which bind antigen and interact with TCR. Second, Pj^{CHO}Ag may be directly displayed on the cell membrane alone or in association with an antigen-binding molecule that serves only to concentrate the antigen but does not interact with TCR. Interestingly, DCs, which are the most efficient APC for protein antigens (29), are rather ineffective in presenting Pj^{CHO}Ag, suggesting that they may lack this putative antigen-presenting/binding molecule.

Recognition of Pj^{CHO}Ag by CD8$^+$ T cell clones is mediated via TCR as shown by the kinetics of TCR triggering which is comparable to that of conventional TCR ligands such as peptide–MHC complexes (30). Moreover the presence of a conserved motif in the CDR3 region of the Vβ chain suggests the existence of a precise structural require-

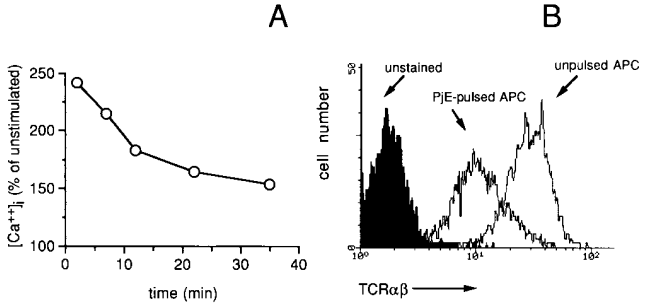

Figure 7. Pj^{CHO}Ag triggers the TCR as conventional TCR ligand. (*A*) Time course of $[Ca^{2+}]_i$ increase in T cell clone P4.2 conjugated with PjE-pulsed B-LCL. (*B*) TCR downregulation in T cells conjugated with unpulsed APC or PjE-pulsed APC.

ment for $Pj^{CHO}Ag$ recognition (30). This result, together with the fact that polyclonal $V\beta8^+$ lines do not respond to $Pj^{CHO}Ag$, rules out the possibility that $Pj^{CHO}Ag$ may act as a superantigen.

The finding that $CD8^+$ T cell clones could be isolated from both atopic patients and from healthy donors is not surprising since they both are naturally exposed to this environmental allergen (31). However, it is interesting that only the clones from the atopic patient produce high levels of IL-4 and IL-5 and express CD40L , suggesting that these $CD8^+$ cells may have undergone a Th2-like polarization in vivo (32, 33). The functional significance of these $CD8^+$ T cells in allergy remains a matter of speculation (34). It is possible that these cells may participate in the production of IL-4 thus influencing the differentiation of naive allergen-specific T cells towards Th2 (35–37). In addition, the requirement for processing and the stability of the epitope suggest the possibility that $CD8^+$ $Pj^{CHO}Ag$-specific T cell clones may function as typical carrier-specific T cells. Interestingly, these clones are not cytotoxic (Sallusto, F., unpublished data) and may thus provide cognate help to antigen-specific B lymphocytes. If this is a general case, the polarization of the specific $CD8^+$ cells towards help or cytotoxicity may become a critical factor controlling the stimulation of an IgE response or its suppression (38, 39).

These results suggest that the response to carbohydrate antigens may be part of the normal response to environmental allergens and that this response can be polarized in atopic patients. Polysaccharides are generally considered typical T-independent antigens. However, T cells that modulate responses to polysaccharides have been observed in several systems (40–46). Our results might explain these earlier observations and raise the possibility that T cell recognition of carbohydrates may play a role in the immune response.

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References

1. Porcelli, S.A., C.T. Morita, and R.L. Modlin. 1996. T cell recognition of non-peptide antigens. *Curr. Opin. Immunol.* 8: 510–516.
2. Porcelli, S.H., C.T. Morita, and M.B. Brenner. 1992. CD1b restricts the response of human $CD4^-CD8^-$ T lymphocytes to a microbial antigen. *Nature (Lond.).* 360:593–596.
3. Beckman, M., S.A. Porcelli, C.T. Morita, S.M. Behar, T. Furlong, and M.B. Brenner. 1994. Recognition of a lipid antigen by CD1-restricted $\alpha\beta^+$ T cells. *Nature (Lond.).* 372: 691–694.
4. Sieling, P.A., D. Chatterjee, S.A. Porcelli, T.I. Prigozy, T. Soriano, M.B. Brenner, M. Kronenberg, P.J. Brennan, and R.L. Modlin. 1995. CD1-restricted T cell recognition of microbial lipoglycans. *Science (Wash. DC).* 269:227–230.
5. Constant, P., F. Davodeau, M.-A. Peyrat, Y. Poquet, G. Puzo, M. Bonneville, and J.-J. Fournié. 1994. Stimulation of human γδ T cells by nonpeptidic mycobacterial ligands. *Science (Wash. DC).* 264:267–270.
6. Morita, C.T., E.M. Beckman, J.F. Bukowski, Y. Tanaka, H. Band, B.R. Bloom, D.E. Golan, and M.B. Brenner. 1995. Direct presentation of nonpeptide prenyl pyrophosphate antigens to human γδ T cells. *Immunity* 3:495–507.
7. Burk, M.R., L. Mori, and G. De Libero. 1995. Human $V\gamma9$-$V\delta2$ cells are stimulated in a cross-reactive fashion by a variety of phosphorylated metabolites. *Eur. J. Immunol.* 25:2052–2058.
8. Harding, C.V., J. Kihlberg, M. Elofsson, G. Magnusson, and E.R. Unanue. 1993. Glycopeptide bind MHC molecules and elicit specific T cell responses. *J. Immunol.* 151:2419–2425.
9. Haurum, J.S., G. Arsequell, A.C. Lellouch, S.Y.C. Wong, R.A. Dweck, A.J. McMichael, and T. Elliott. 1994. Recognition of carbohydrate by major histocompatibility complex class I-restricted, glycopeptide-specific cytotoxic T lymphocytes. *J. Exp. Med.* 180:739–744.
10. Michaelsson, E., V. Malmsdtrom, S. Reis, A. Engstrom, H. Burkhardt, and R. Holmdahl. 1994. T cell recognition of carbohydrates on type II collagen. *J. Exp. Med.* 180:745–749.
11. Dudler, T., F. Altmann, J.M. Carballido, and K. Blaser. 1995. Carbohydrate-dependent, HLA class II-restricted, human T cell response to bee venom allergen phospholipase A2 in allergic patients. *Eur. J. Immunol.* 25:538–542.
12. Martin, S., and H.U. Weltzien. 1994. T cell recognition of haptens, a molecular view. *Int. Arch. Allergy Immunol.* 104: 10–16.
13. Ishioka, G.Y., A.G. Lamont, D. Thomson, N. Bulbow, F.C.A. Gaeta, A. Sette, and H.M. Grey. 1992. MHC interaction and T cell recognition of carbohydrate and glycopeptide. *J. Immunol.* 148:2446–2451.
14. Harding, C.V., R.W. Roof, P.M. Allen, and E.R. Unanue. 1991. Effect of pH and polysaccharides on peptide binding to class II major histocompatibility complex molecules. *Proc. Natl. Acad. Sci. USA.* 88:2740–2744.
15. D'Amato, G., and G. Lobefalo. 1989. Allergenic pollens in the southern Mediterranean area. *J. Allergy Clin. Immunol.* 83:

116–122.
16. Sallusto, F., S. Corinti, C. Pini, M.M. Biocca, G. Bruno, and G. Di Felice. 1996. *Parietaria judaica*-specific T-cell clones from atopic patients: heterogeneity in restriction, Vβ usage, and cytokine profile. *J. Allergy Clin. Immunol.* 97:627–637.
17. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony–stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J. Exp. Med.* 179:1109–1118.
18. Lanzavecchia, A. 1985. Antigen-specific interaction between T and B cell. *Nature (Lond.).* 314:537–539.
19. Elsasser-Beile, U., S. von Kleist, and H. Gallati. 1991. Evaluation of a test system for measuring cytokine production in human whole blood cell cultures. *J. Immunol. Methods* 139: 191–195.
20. Giuliani, A., C. Pini, S. Bonini, N. Mucci, L. Ferroni, and G. Vicari. 1987. Isolation and purification of a major allergen from *Parietaria officinalis* pollen. *Allergy*. 42:434–440.
21. Bradford, M.M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
22. Edge, A.S.B., C.R. Faltinek, L. Hof, L.E. Reichert, Jr., and P. Weber. 1981. Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. *Anal. Biochem.* 118:131–137.
23. Spiro, R.G. 1967. Studies on the renal glomerular basement membrane. Nature of the carbohydrate units and their attachment to the peptide portion. *J. Biol. Chem.* 242:1943–1948.
24. Ashwell, G. 1966. New colorimetric methods of sugar analysis. *In* Methods in Enzymology. S.P. Colowick and N.O. Kaplan, editors. Academic Press Inc., New York. VIII:93–95.
25. Valitutti, S., M. Dessing, K. Aktories, H. Gallati, and A. Lanzavecchia. 1995. Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. *J. Exp. Med.* 181:577–584.
26. De Palma, R., and J. Gorsky. 1995. Restricted and conserved T-cell repertoire involved in allorecognition of class II major histocompatibility complex. *Proc. Natl. Acad. Sci. USA*. 92: 8836–8840.
27. Pamer, E.G., C.-R. Wang, L. Flaherty, K. Fischer-Lindahl, and M.J. Bevan. 1992. H-2M3 presents a *Listeria monocytogenes* peptide to cytotoxic T lymphocytes. *Cell.* 70:215–223.
28. Nataraj, C., M.L. Brown, R.M. Poston, S.M. Shawar, R.R. Rich, K. Fisher Lindahl, and R.J. Kurlander. 1996. H2-M3wt-restricted, *Listeria monocytogenes*-specific CD8 T cells recognize a novel, hydrophobic, protease-resistant, periodate-sensitive antigen. *Int. Immunol.* 8:367–378.
29. Steinman, R.M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* 9:271–295.
30. Valitutti, S., S. Muller, M. Cella, E. Padovan, and A. Lanzavecchia. 1995. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature (Lond.).* 375:148–151.
31. Di Felice, G., A. Mari, N. Mucci, C. Afferni, G. Bruno, and C. Pini. 1989. T cell responses to a *Parietaria judaica* pollen extract: comparison between *Parietaria*-sensitive patients, other atopics and healthy controls. *Allergy*. 44:322–329.
32. Salgame, P., J.S. Abrams, C. Clayberger, H. Goldstein, J. Convit, R.L. Modlin, and B.R. Bloom. 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science (Wash. DC).* 254:279–281.
33. Sad, S., R. Marcotte, and T.R. Mosmann. 1995. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. *Immunity*. 2:271–279.
34. Romagnani, S. 1994. Lymphokine production by human T cells in disease state. *Annu. Rev. Immunol.* 12:227–257.
35. Yoshimoto, T., A. Bendelac, J. Hu-Li, and W.E. Paul. 1995. Defective IgE production by SJL mice is linked to the absence of CD4+, NK1.1+ T cells that promptly produce interleukin 4. *Proc. Natl. Acad. Sci. USA*. 92:11931–11934.
36. Maggi, E., M.G. Giudizi, R. Biagiotti, F. Annunziato, R. Manetti, M.-P. Piccinni, P. Parronchi, S. Sampognaro, L. Giannarini, G. Zuccati, et al., 1994. Th2-like CD8+ T cells showing B cell helper function and reduced cytolytic activity in human immunodeficiency virus type I infection. *J. Exp. Med.* 180:489–495.
37. Paganelli, R., E. Scala, I.J. Ansotegui, C.M. Ausiello, E. Halapi, E. Fanales-Belasio, G. D'Offizi, I. Mezzaroma, F. Pandolfi, M. Fiorilli, et al., 1995. CD8+ T lymphocytes provide helper activity for IgE synthesis in human immunodeficiency virus-infected patients with hyper-IgE. *J. Exp. Med.* 181: 423–428.
38. Bloom, B.R., R.L. Modlin, and P. Salgame. 1992. Stigma variations: observations on suppressor T cells and leprosy. *Annu. Rev. Immunol.* 10:453–487.
39. Seder, R.A., and G.G. Le Gros. 1995. The functional role of CD8+ T helper type 2 cells. *J. Exp. Med.* 181:5–7.
40. Baer, H., and S.D. Chaparas. 1964. Tuberculin reactivity of a carbohydrate component of unheated BCG culture filtrate. *Science (Wash. DC).* 146:245–247.
41. Baker, P.J., P.W. Stashak, D.F. Amsbaugh, B. Prescott, and R.F. Barth. 1970. Evidence for the existence of two functionally distinct types of cells which regulate the antibody response to type III pneumococcal polysaccharide. *J. Immunol.* 105:1581–1586.
42. Shapiro, M.E., A.B. Onderdonk, D.L. Kasper, and R.W. Finberg. 1982. Cellular immunity to *Bacteroides fragilis* capsular polysaccharide. *J. Exp. Med.* 155:1188–1197.
43. Bellamy, A., A.N. Davison, and M. Feldmann. 1986. Derivation of ganglioside-specific T cell lines of suppressor or helper phenotype from cerebrospinal fluid of multiple sclerosis patients. *J. Neuroimmunol.* 12:107–120.
44. Katsuki, M., K. Kakimoto, S. Kawata, S. Kotani, and T. Koga. 1987. Induction of delayed-type hypersensitivity by the T cell line specific to bacterial peptidoglycans. *J. Immunol.* 139:3570–3572.
45. Henningsson, C.M., S. Selvaraj, G.D. MacLean, M.R. Suresh, A.A. Noujaim, and B.M. Longenecker. 1987. T cell recognition of a tumor-associated glycoprotein and its synthetic carbohydrate epitopes: stimulation of anti-cancer T cell immunity in vivo. *Cancer Immunol. Immunother.* 25:231–241.
46. Moll, H., G.F. Mitchell, M.J. McConville, and E. Handman. 1989. Evidence for T-cell recognition in mice of a purified lipophosphoglycan from *Leishmania major*. *Infect. Immun.* 57: 3349–3356.