

Short paper

A flavonoid sulfate antigen activates human $\alpha\beta$ CD8⁺ Th2 lymphocytes in pollen allergyFederica Sallusto¹, Rémy Poupot², Myriam Clergue², Rafaele DePalma³ and Jean-Jacques Fournié²¹ Basel Institute for Immunology, Basel, Switzerland² INSERM 395 & IFR 30, CHU Purpan, Toulouse, France³ Fondazione Salvatore Maugeri, Pavia, Italy

Cellular immune responses are initiated when T lymphocytes expressing $\alpha\beta$ TCR recognize peptide antigens bound to MHC molecules or, less frequently, double-stranded glycolipid antigens bound to CD1 molecules. In the allergy to *Parietaria judaica*, human $\alpha\beta$ CD8⁺ Th2 lymphocytes react to a non-peptide pollinic antigen presented by B cells. The environmental allergen was purified and identified as a new flavonoid pigment: 2'-O-sulfate, 6-O- β -D-glucuronopyranosyl, 2',5,6-trihydroxy-isoflavone. Its specific recognition by $\alpha\beta$ CD8⁺ Th2 T cells (1) depends upon an MHC- and CD1-independent presentation mediated by B cells, (2) is determined by the flavonoid carbohydrate and sulfate groups and (3) leads to positive skin prick test in allergic patients. Hence, an unusual mode of aromatic sulfated antigen recognition by $\alpha\beta$ CD8⁺ Th2 T lymphocytes might underlie the cellular mediation of human allergy to plant allergens.

Key words: Allergy / Antigen / Sulfated flavonoid / T lymphocyte

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1 Introduction

There is growing evidence that T cell responses can be initiated upon recognition of antigens distinct from peptides bound to classical MHC molecules. Two structural types of nonpeptide antigens are known so far. While a first type comprises small hydrophilic phosphoantigens recognized by human $\gamma\delta$ T cells without antigen-presenting molecule [1–3], a second type involves two-stranded aliphatic antigens which, upon presentation by CD1 molecules, are recognized by invariant human $\alpha\beta$ T or murine NKT cells (for review see [4]). Recently it was shown that $\alpha\beta$ CD8⁺ T cell clones drawn from PBL of patients allergic to *Parietaria judaica*, a common environmental allergen of the Mediterranean area [5], proliferate and release Th2 cytokines when stimulated by *P. judaica* pollen extract (PjE) [6]. As this T cell response only occurs in the presence of APC but independently of MHC class I or CD1 molecules, and is abrogated upon sodium periodate oxidation of PjE, recognition of a non-peptide antigen (so-called PjCHO) was inferred [6].

Here we report complete purification and structural characterization of PjCHO. This $\alpha\beta$ T cell ligand differs structurally from classical peptide and double-stranded aliphatic $\alpha\beta$ T cell antigens. We show that triggering $\alpha\beta$ CD8⁺ T cell clone responses by purified PjCHO involves MHC- and CD1-independent presentation.

2 Results and discussion

The proliferation of PjE-reactive CD8⁺ T cell clones served as readout to monitor the purification of PjCHO from PjE. As the antigen is hydrophilic [6], the extract was delipidated and the aqueous phase further separated by anion exchange chromatography. All the initial T cell-stimulating bioactivity was recovered in anionic fractions together with 90 % of the total dry mass, which comprised brown pigments, carbohydrates, proteins and nucleic acids (not shown). The most active fraction (0.3 M CH₃COO[−] NH₄⁺) composed of di- to penta-anionic molecules was further separated by preparative C₁₈-HPLC; fractions were collected and assayed for proliferation of the T cell clone (Fig. 1, left). The bioactive HPLC fractions (no. 21–34) were retained by an ion-pairing eluent, but eluted with an acetonitrile in water gradient prior to inactive brown pigments, indicating an amphiphilic anion structure. The same procedure was repeated,

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Abbreviations: MS: Mass spectrometry PjE: *Parietaria judaica* pollen extract

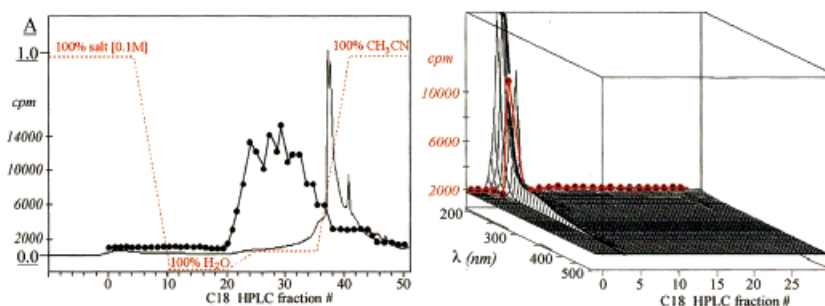


Fig. 1. Isolation of PjCHO. (Left) C₁₈-HPLC separation of the bioactive fraction from PjE (UV at 268 nm: black line; eluent: red broken line). Fractions (1 min) were tested for stimulation of the PjE-reactive P4.2 clone (black dots). (Right) Analytical C₁₈-HPLC separation of pooled fractions no. 21–34 monitored by photodiode array and tested for stimulation of the P4.2 clone (red dots).

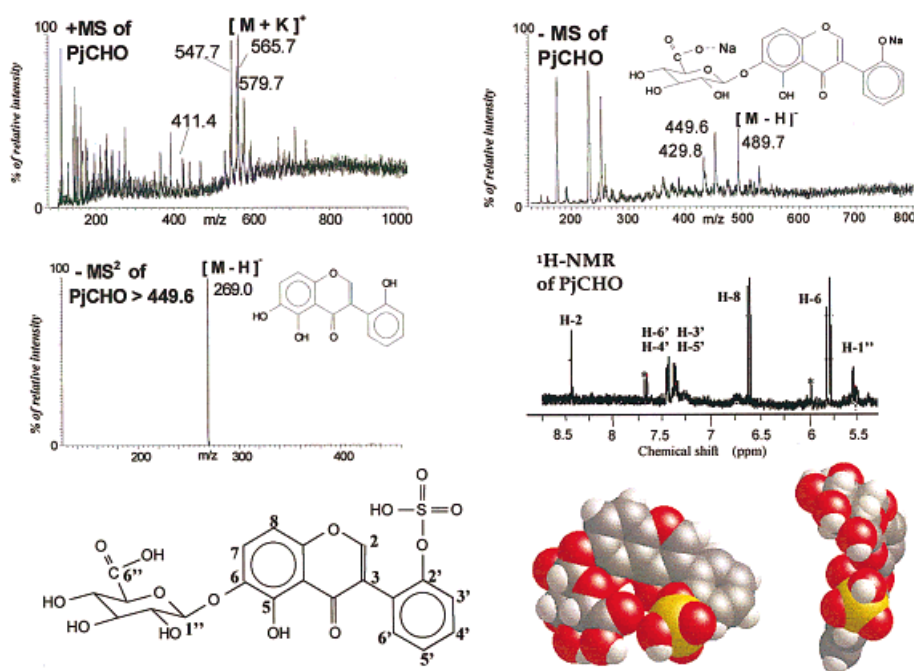


Fig. 2. Structural identification of PjCHO. (Upper left) Electrospray-ionization MS of PjCHO [(+) mode] showed m/z 565 and m/z 547: K^+ adduct of PjCHO ($M+K$)⁺ and its anhydro derivative ($M-H_2O+K$)⁺ (molecular mass of PjCHO = 526), as well as a fragment due to glucuronic acid loss at m/z 411 (M -glucuronic acid+ $K+Na$)⁺. (Upper right) MS¹ of PjCHO [(-) mode] showed no molecular ion, but a desulfated fragment at m/z 489 (M -sulfate+2Na-3H)⁻ and its related anhydro derivative at m/z 449 (M -sulfate- $H_2O+Na-2H$)⁻. (Lower left) MS² of m/z 449 gives m/z 269, corresponding to a tri-hydroxylated isoflavone aglycon [15]. The same MS² experiment with genistin yielded also an m/z 269 genistein ion [15] (not shown). (Lower right) The 5.5–8.5 ppm region from the ¹H – nuclear magnetic resonance spectrum (400 MHz, D₂O) of PjCHO confirms 2',5,6-trihydroxylated isoflavone [16] (H-2, δ 8.41 ppm, s; H-7, δ 5.77 ppm, d; 12 Hz; H-8, δ 6.60 ppm, d; 12 Hz), and evidences the β -O-glucuronyl (H-1'', δ 5.54 ppm, d; 5.6 Hz; H-5'', δ 4.1 ppm, d; 7 Hz). Four overlapping phenolic ring protons (δ 7.44 ppm, d; δ 7.42 ppm, t; δ 7.38 ppm, t; δ 7.36 ppm, d) which couple together (2-D correlation spectroscopy ¹H-¹H – nuclear magnetic resonance, not shown), harbor the splittings of datiscetin, a flavonol with 2'-OH instead of 4'-OH [16]. Their downfield shift (+0.4 ppm) as compared to genistin evidences a 2'-O-substitution by an electron-withdrawing group as sulfate [11, 13]. (Below) Tridimensional model of PjCHO. O: red, S: yellow (Alchemy 2000, Tripos, St Louis, MO).

without ion-pairing, on these pooled bioactive fractions and yielded a single peak [retention time (Rt) 4.0] of purified PjCHO (< 1 mg) that accounted for the total PjE bioactivity on the PjE-reactive CD8⁺ T cell clone (Fig. 1, right).

The optical spectrum of PjCHO in water (Fig. 1, right) (band I: shoulder at 330–340 nm, band II: λ_{max} 268 nm), identical to that of synthetic genistin disulfate (Rt 4.0, not shown), and its blue fluorescence with ammonium (λ_{exc} 360 nm, λ_{em} 480 nm, not shown) are characteristic of a flavonoid [7, 8]. This compound appears as a yellow-orange spot under UV light on a 2-D paper chromatography (retention factors: Rf₁ 0.15, Rf₂ 0.95; not shown) or on a silica gel thin layer chromatography (Rf 0.55, not shown) sprayed with Neu reagent [9], so PjCHO is most likely a dianionic glycosylated isoflavone [10]. Its UV spectral analysis in methanol (identical to Fig. 1, left) and the HCl-resistant AlCl₃-induced bathochromic shift (+70 nm) of band I (shoulder, not shown) indicated a 5-OH isoflavone [7, 8, 10].

Analytical HPLC indicated that PjCHO (Rt 3–4 min) is a glycosylated and monosulfated isoflavone, related to standards of genistin (7-O- β D-glucopyranosyl-genistein) disulfate or rutin (3-O-[α L-rhamnopyranosyl (1 $\rightarrow$ 6) β D-glucopyranosyl]-quercetin) disulfate, but neither to the sulfated aglycones genistein (4',5,7-trihydroxy-isoflavone) 4',7 disulfate or quercetin (3,3',4',5,7-pentahydroxy-flavone) 4',7 disulfate (Rt 7–10 min), nor the nonsulfated flavonoid glycosides genistin or rutin (Rt 25–27 min) and the aglycon flavonoids genistein or quercetin (Rt > 30 min). Further, mild acid hydrolysis (HCl 1N, 45°C, 1 h) of PjCHO failed to release intermediates detected by HPLC while strong acidic conditions (HCl 2N, 85°C, 15 min) destroyed it. Together, the above data indicated that PjCHO was a sulfated isoflavone uronide [11].

The structure of PjCHO was finally determined by ion-trap electrospray-ionization mass spectrometry (MS), ¹H-nuclear magnetic resonance (Fig. 2) and enzymatic degradation with an aryl sulfatase [11]. As PjCHO bears 5-OH, its complete structure is: 2'-O-sulfate, 6-O- β D-glucuronopyranosyl, 2',5,6-trihydroxy-isoflavone (Fig. 2). To our knowledge, no such isoflavonoid has been reported yet, as only glucuronylated and sulfated flavones have been detected by thin layer chromatography in Australian cypress [11].

PjE-specific T cell clones only proliferate and release Th2 cytokines in response to PjE once live and unfixed APC such as B cells are provided [6]. As shown in Fig. 3A, CD8⁺ Th2 T cell recognition of purified PjCHO requires a presentation by unfixed presenting molecules and live APC. T cells showed a PjCHO dose-dependent prolifera-

tion and IFN- γ production (not shown) in the presence of live EBV-transformed lymphoblastoid cell lines cells or with the β 2-microglobulin-deficient Daudi cell line. Thus neither MHC class I nor CD1 or any other β 2-microglobulin-associated molecules are involved in the presentation of PjCHO. The recognition of PjCHO by the $\alpha\beta$ CD8⁺ Th2 cell clones takes place via its hydrophilic parts (Fig. 3 C–E): the T cell response was abrogated by PjCHO treatment with aryl sulfatase or with sodium periodate, which remove the sulfate group or oxidize the 6-O- β D-glucuronide into a carboxylic ether, respectively. As several synthetic flavonoids representing potential degradative PjCHO byproducts (genistin 4',6"-disulfate, genistein 4',7-disulfate, genistin and several other flavonoid sulfates) did not stimulate the $\alpha\beta$ CD8⁺ T cells (Fig. 3 B, F), little degradative processing of PjCHO might precede its recognition by the T cells. Together, these results suggest that the hydrophilic and anionic 2'-O-sulfate and 6-O- β D carboxylic glycoside substituting the aromatic core are essential determinants of PjCHO recognition by $\alpha\beta$ T cells. Its small size (16.7 Å length/5.3 Å height, Fig. 2), makes TCR engagement by PjCHO alone unlikely, suggesting the involvement of an antigen-presenting molecule distinct from MHC and CD1 [6].

In contrast with the well-known T cell-suppressive properties of flavonoids [12], PjCHO is a novel isoflavonoid characterized by its capacity to stimulate *in vitro* specific human $\alpha\beta$ CD8⁺ Th2 lymphocyte responses. The functional significance of these observations was assessed *in vivo* by PjCHO skin prick test on patients allergic to *P. judaica* (Fig. 4). Both PjE and purified PjCHO provoked an allergic response in a few minutes. Thus PjCHO-specific $\alpha\beta$ CD8⁺ Th2 lymphocytes are part of the immune allergic response to the environmental allergenic plant *P. judaica*.

Beside double-stranded glycolipids, our results evidence a new type of non-peptide antigen (so-called sulfantigen) recognized by $\alpha\beta$ CD8⁺ T cells. Recognition of this antigenic allergen is mediated by sulfate and glucuronate, both negatively charged groupments, and might to some extent resemble that of phosphoantigens by human $\gamma\delta$ T cells.

3 Materials and methods

3.1 Cell culture

$\alpha\beta$ CD8⁺ T cell clones P4.2 and P4.19 were isolated and characterized as already described [6]. For antigen presentation assays, 5×10^4 cells of clone P4.19 were cultured with 5×10^4 live allogeneic EBV-transformed lymphoblastoid cell lines or Daudi cells, which had been treated with 1 mg/ml

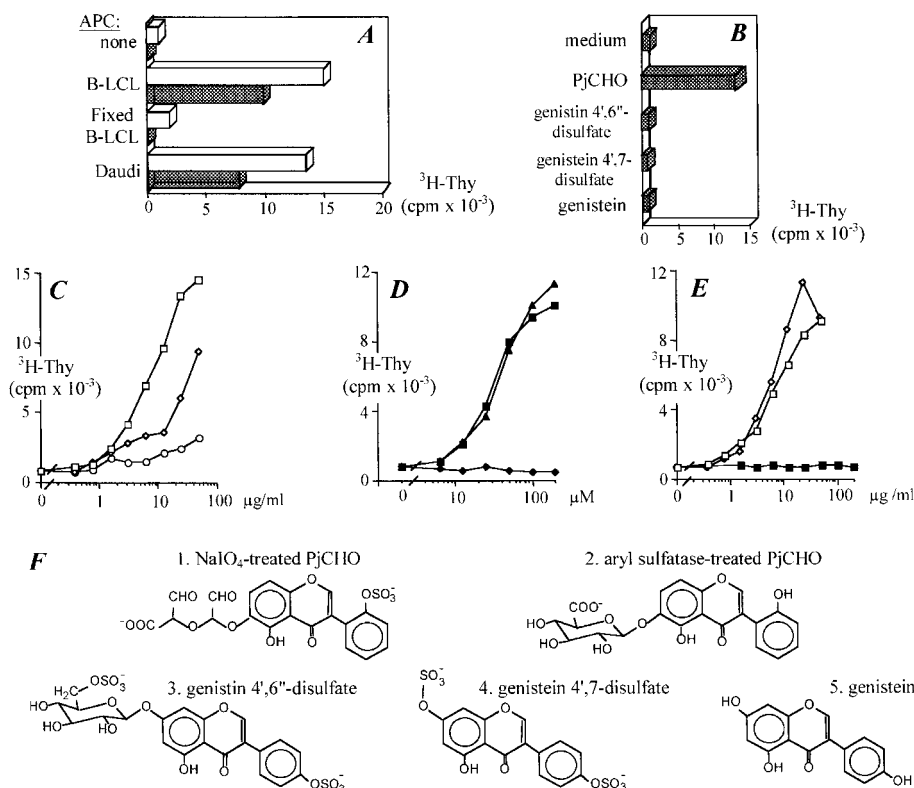


Fig. 3. MHC-unrestricted and PjCHO-selective reactivity of the $\alpha\beta$ CD8 $^+$ T cell clone P4.19. (A) P4.19 clone proliferation in response to 50 $\mu\text{g/ml}$ PjE (□) or to 50 μM purified PjCHO (■) without or with APC (fixed or not). (B) P4.19 clone proliferation in response to 100 μM PjCHO-related flavonoids. (C) Stimulation of P4.19 clone by PjE (□), aryl sulfatase-treated PjE (◇) or NaIO_4 -treated PjE (○). (D) Stimulation of P4.19 clone by PjCHO (■), mock-PjCHO (▲) and aryl sulfatase-treated PjCHO (◆). (E) Stimulation of a PjCHO-unrelated PjE-reactive $\alpha\beta$ CD4 $^+$ T cell clone from the same donor with PjE (□), sulfatase-treated PjE (◇) and PjCHO (■, in μM). (F) Structures of the molecules used for the tests.

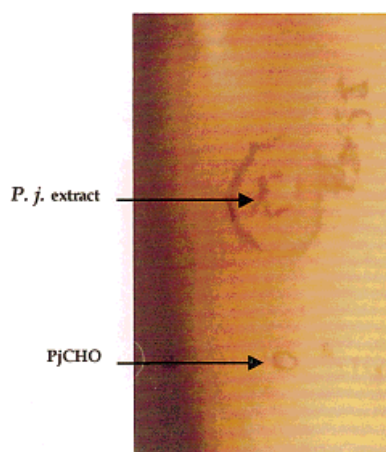


Fig. 4. PjCHO in skin prick test. Wheals and flares were marked with a dermatographic pencil and photographed on an allergic patient's forearm 20 min after the prick.

mitomycin C (Sigma Chemical Co., St Louis, MO) for 45 min at 37 °C in 200 μl complete medium supplemented with 10 % FCS (Life Technologies, Gaithersburg, MD). Cultures were pulsed with 1 μCi [^3H] thymidine (2 Ci/mmol; Amersham International, Little Chalfont, GB) on day 2 and harvested 16 h later. Cells were fixed with 0.05 % glutaraldehyde for 30 s and blocked with 0.1 M lysine in PBS for 5 min before washes. Proliferative response was measured at day 3 of culture by [^3H] thymidine incorporation as above. P4.2 cells (5×10^4) were cultured with 5×10^4 mitomycin C-treated MHC-unrelated EBV-transformed lymphoblastoid cell lines and tested fractions were diluted 1:100.

Desulfated PjE or PjCHO were obtained by incubation with 1U *Aerogenes aerogenes* sulfohydrolase (Sigma Chemical Co.) for 4 h at 37 °C in 10 mM Tris pH 7.2.

3.2 Separation and purification

After delipidation, PjE was separated by anion exchange chromatography on a DEAE column. Elution was performed with H₂O, 0.1 M, 0.3 M and 1 M CH₃COO⁻ NH₄⁺ [5 void volumes (V₀) each].

HPLC was performed on C₁₈ columns (250 × 20 mm Ultra-sep 6 μ for preparative HPLC and 250 × 4 mm Nucleosil 5 μ for analytical HPLC; both from Bischoff, Germany) with photodiode array detection (PDA 996 Millennium Software; Waters Millipore, Milford, MA). Eluent sequences were: 0–15 min: (ion-pairing) CH₃COO⁻ NH₄⁺ 0.1 M; 15–20 min: H₂O gradient 0 to 100 %; 20–30 min: 100 % H₂O; 30–35 min: CH₃CN gradient 0 to 10 %; 35–45 min: 10 % CH₃CN in H₂O; 45–50 min: CH₃CN gradient 10 to 100 %; 50 to 60 min: 100 % CH₃CN for preparative HPLC (5 ml/min); and: 0–15 min: 100 % H₂O; 15–20 min: CH₃CN gradient 0 to 10 %; 20–25 min: 10 % CH₃CN in H₂O; 25–30 min: CH₃CN gradient 10 to 30 %; 30–35 min: CH₃CN gradient 30 to 100 % for analytical HPLC (0.8 ml/min).

2-D paper chromatography was performed with solvent 1: *tert*-butyl alcohol/acetic acid/water (3/1/1), then solvent 2: 15 % acetic acid in water; and silica gel thin layer chromatography was performed with isopropanol/formic acid/water (4/1/1) as solvent.

3.3 Synthesis of sulfated flavonoids and analytical methods

Flavonoid disulfate standards were synthesized essentially as described [13], using diisopropylcarbodiimide (PerSeptive Biosystems, Hamburg, Germany) as coupling agent and separation of sulfated products by Sep-Pak QMA cartridges (Waters Millipore) eluted with 0.5 M triethylammonium bicarbonate buffer, pH 7.2 (Sigma Chemical Co.), prior to lyophilization and control by analytical HPLC.

Mass spectra were recorded on an ion-trap LCQ mass spectrometer (Finnigan Corporation, San Jose, CA). 1- and 2-D correlation spectroscopy ¹H – nuclear magnetic resonance spectra were measured on a Brücker 400 MHz spectrometer (Brücker, Karlsruhe, Germany).

3.4 Skin prick test for PjE and PjCHO

After a gentle skin scratching with a fine needle, a drop (about 20 μl) of PjE (Neoabello, Madrid, Spain) was put on the forearm of the patient and a 2 mm point lancet (DHS Bayrofarm, Milan, Italy) was used for the prick, according to standard protocols [14]. Following the procedure, purified PjCHO was tested at 50 μM in PBS. As control, we used fractions that were not able to stimulate T cells. After 20 min the wheal and the flare were marked using a dermatographic pencil and a picture was taken using a Nikon camera with a 80 mm lens.

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