

The lipophilic hapten parthenolide induces interferon- γ and interleukin-13 production by peripheral blood-derived CD8 $^{+}$ T cells from contact allergic subjects *in vitro*

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Summary

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Conflicts of interest

H.W., K.M., B.Z. and N.A. are or have been employees at Mabtech AB, a Swedish biotech company from which monoclonal antibody reagents used in the study were obtained. No author owns shares or stocks in the company.

H.W. and K.M. contributed equally to the study.

Background Peripheral blood mononuclear cells (PBMC) from persons with contact allergy to nickel react *in vitro* predominantly with nickel-induced CD4 $^{+}$ T cell-mediated production of both T-cell type 1 and 2 cytokines.

Objectives The aim of the present study was to investigate if the contact allergen parthenolide, a sesquiterpene lactone of lipophilic character, elicits an immune response which differs from that induced by water-soluble nickel ions.

Patients and methods Ten allergic subjects with strong ($n = 6$), moderate ($n = 2$), or weak ($n = 2$) patch-test reactivity to parthenolide and five patch test-negative control subjects participated in the study. PBMC from the subjects were analysed for *in vitro* reactivity with parthenolide by an enzyme-linked immunospot (ELISpot) assay, measuring cytokine production at the single-cell level.

Results The allergic group, but not the control group, responded to parthenolide with increased numbers of cells producing interferon (IFN)- γ , interleukin (IL)-2, IL-4, IL-5 ($P < 0.05$ for all) and IL-13 ($P < 0.01$). The responses manifested by T-cell type 1 (IFN- γ and IL-2) and type 2 (IL-4, IL-5 and IL-13) cytokines were positively correlated between cytokines. Subjects with a strong or moderate, but not weak or negative, patch-test reaction displayed detectable *in vitro* responses. In contrast to the CD4 $^{+}$ T cell-mediated peripheral reactivity induced by nickel, cell depletion experiments identified the parthenolide-reactive IFN- γ - and IL-13-producing cells as CD8 $^{+}$ T cells.

Conclusions The finding that the PBMC reactivity to parthenolide in humans involves a CD8 $^{+}$ T cell-mediated type 1 and 2 cytokine response warrants further studies on the relationship between the chemical nature of a hapten and the resulting immune response.

Allergic contact dermatitis (ACD) is the eczematous manifestation of a contact allergic reaction (a delayed-type hypersensitivity reaction) in which the causative agents are low-molecular-weight substances, i.e. haptens, to which the skin is exposed. The nature and chemical properties of these haptens differ; some are metal ions while others are organic compounds used as, or included in, for example, preservatives, fragrances, rubbers, plastics and topical antibiotics.¹

Studies on the immunological mechanisms involved in ACD in humans, are often based on reactivity to nickel, the most common cause of ACD. Assessment of the *in vitro* response to the metal ion nickel, in peripheral blood mononuclear cells (PBMC) from humans with nickel allergy, has revealed a cyto-

kine profile involving T-cell type 1 [e.g. interleukin (IL)-2 and interferon (IFN)- γ] and type 2 cytokines (e.g. IL-4, IL-5 and IL-13),^{2–5} although the involvement of IFN- γ has not been demonstrated in all studies.^{6–8} In addition, a mixed type 1 and 2 profile has been described *in vitro* in PBMC from subjects with contact allergy to other metals such as cobalt, palladium, chromium and gold.⁹ It has further been shown that CD4 $^{+}$ T cells are responsible for both the type 1 and 2 cytokine production detected in PBMC from nickel-sensitized individuals.^{5,10}

Less is known about the peripheral immunological reactivity induced by organic haptens, comprising the vast majority of the haptens that can cause contact allergy in humans. Haptens

such as methylchloroisothiazolinone/methylisothiazolinone (MCI/MI) and fragrance mix have been shown to elicit a cytokine profile, similar to that described for nickel, in PBMC from allergic persons, but the T cells involved have not been defined.^{4,11} Similarly to CD4+ T cells which can be divided into type 1 and 2 cells [i.e. T helper (Th) 1 and Th2] based on their cytokine production, CD8+ T cells can differentiate into cytotoxic (Tc) 1 and Tc2 cells.¹² Thus, although a similar cytokine response profile may be observed in PBMC reactive with different haptens, the T cells responsible for the cytokine production may not necessarily be of the same phenotype. To characterize more completely the mechanisms involved in the immune response to different haptens, it is also of relevance to define the T cells involved.

The sesquiterpene lactone parthenolide, the hapten investigated in this study, is a lipophilic organic compound with a partition coefficient (logP) of 2.4. Many organic haptens are of a lipophilic nature, which may be of specific relevance for the immune responses elicited (see Discussion).¹³ Parthenolide is found in the perennial herb feverfew, *Tanacetum parthenium*, which belongs to the Compositae family and is in addition found in at least 33 other species including some belonging to other genera.^{14,15} Parthenolide has anti-inflammatory and toxic effects and is traditionally used for prevention of migraine.^{16,17} Besides this, parthenolide has also been shown to be a sensitizer in guinea pig experiments¹⁸ and can cause ACD in exposed humans.^{19,20} Subjects with ACD to feverfew or other Compositae plants usually have positive patch-test reactions to plant extracts, sesquiterpene lactone mix and/or parthenolide.¹⁹ Compositae-induced dermatitis affects 0.7–1.4% of the general population and allergy to Compositae mix or the sesquiterpene lactone mix is among the 10–20 most common causes of contact sensitization in Europe.^{13,21} Parthenolide yields a positive patch test in approximately 75% of patients positive for sesquiterpene lactone mix.²²

The aim of the present study was to investigate if a hapten consisting of a lipophilic organic molecule elicits an immune response that, in terms of the cytokine profile and the phenotype of the cytokine-producing cells, differs from the immune response previously described to be induced by nickel.

Materials and methods

Subjects

Ten subjects allergic to parthenolide with strong (+++, $n = 6$), moderate (++, $n = 2$) and weak (+, $n = 2$) patch-test reactivity to parthenolide and five patch test-negative control subjects without any history of ACD from parthenolide participated in the analysis of the cytokine profile elicited by parthenolide; the patch-test data for these subjects were not disclosed until after completion of the *in vitro* analysis. The subjects were, with one exception (subject 6), all female with a mean age of 55 years (range 37–62) and 51 (41–64) for

allergic and control groups, respectively. In addition, two subjects with strong patch-test reactions to parthenolide and three patch test-negative subjects were included in pilot studies. Two further subjects with strong patch-test reactivity to parthenolide were included for the phenotype characterization of cytokine-producing cells; in these experiments a subject with previously established strong patch-test reactivity to 5% NiSO₄ in petrolatum was also included for comparison. The study was approved by the Ethics Committee at the Medical Faculty at Lund University, Lund, Sweden, and all subjects gave their informed consent to participation.

Parthenolide patch test

All subjects were examined by a dermatologist and patch tested with parthenolide 0.1% w/w in petrolatum supplied by Chemotechnique Diagnostics (Vellinge, Sweden). The test preparation was applied on the back with Finn chambers (Epi-test Ltd Oy, Tuusula, Finland) on Scanpor tape (Norgeplaster A/S, Vennsla, Norway) and removed after 48 h. The test reactions were read according to the International Contact Dermatitis Research Group on days 3 and 7.²³

Isolation and stimulation of peripheral blood mononuclear cells

Thirty millilitres of blood from each subject was obtained by venipuncture and collected in sterile, heparinized glass vials. PBMC were isolated and frozen as previously described.³ Prior to analyses, PBMC were thawed at 37 °C and washed twice with cell culture medium: RPMI 1640 medium supplemented with HEPES (10 mmol L⁻¹), L-glutamine (2 mmol L⁻¹), penicillin G sodium (100 U mL⁻¹), streptomycin sulphate (100 µg mL⁻¹) (all from Gibco BRL, Life Technologies Ltd, Paisley, U.K.) and 10% fetal bovine serum (FBS; HyClone Laboratories Inc., Logan, UT, U.S.A.). The cells were left for 1 h at 37 °C in humidified air with 5% CO₂ and were subsequently transferred to new tubes to remove possible cell debris. The cells were counted by Trypan blue exclusion and adjusted to 3.0×10^6 cells mL⁻¹. PBMC cultures in medium with or without parthenolide (P-0667, Sigma Chemical Co., St Louis, MO, U.S.A.), dissolved and diluted in dimethyl sulphoxide (DMSO) prior to addition to the cell cultures, were established in round-bottomed 5-mL Falcon tubes (BD Labware, Franklin Lakes, NJ, U.S.A.). Parthenolide was used at concentrations as indicated elsewhere. DMSO was used at a final concentration of 0.1% in the cell suspensions; pilot tests showed that a DMSO concentration of < 1% did not affect the cellular reactivity to parthenolide. PBMC activated with the polyclonal T-cell activators phytohaemagglutinin (PHA; Oxoid AB, Sollentuna, Sweden) and monoclonal antibody (mAb) to human CD3 (α -CD3-2; Mabtech, Nacka Strand, Sweden) were used as positive controls. The PBMC were cultured in tubes for 18–20 h at 37 °C in humidified air with 5% CO₂ prior to enzyme-linked immunospot (ELISpot) analysis.

Enzyme-linked immunospot assay

Prior to coating, 96-well polyvinylidene difluoride membrane plates (MAIPSWU10; Millipore, Bedford, MA, U.S.A.) were treated with 70% ethanol (50 μL per well) for 1 min and washed five times (200 μL per well) with sterile deionized H_2O . For coating, capture mAbs against IFN- γ (1-D1K), IL-2 (IL2-I), IL-4 (IL4-I), IL-5 (TRFK5) and IL-13 (IL13-I) (Mabtech) at 15 $\mu\text{g mL}^{-1}$ in sterile phosphate-buffered saline (PBS; pH 7.4) were added (100 μL per well). The plates were incubated for 16 h at 4 °C followed by five washes using sterile PBS (200 μL per well) and blocking with cell medium (200 μL per well) for 1 h at room temperature. Subsequently, PBMC cultures pre-incubated with or without stimuli as described above, were transferred in triplicate to the ELISpot wells (100 μL ; 3.0×10^5 cells per well) and left for an additional 18–20 h incubation at 37 °C in humidified air with 5% CO_2 ; pilot tests showed that the pre-incubation of PBMC in vials, followed by incubation in ELISpot plates, yielded a better response to parthenolide compared with incubating the cells directly in the ELISpot plate for 40 h. The wells were emptied and thereafter washed six times with PBS (200 μL per well) in an ELISpot washer (ELx50™ Auto Strip Washer; Bio-Tek Instruments Inc., Winooski, VT, U.S.A.). Then, biotinylated detection mAbs against IFN- γ (7-B6-1-biotin), IL-2 (IL2-II-biotin), IL-4 (IL4-II-biotin), IL-5 (5A10-biotin) and IL-13 (IL13-II-biotin) (Mabtech) at 1 $\mu\text{g mL}^{-1}$ in PBS with 0.5% FBS were added (100 μL per well) and incubated for 2 h at room temperature. The plates were washed again (as above) and streptavidin-alkaline phosphatase conjugate (Mabtech) diluted 1 : 1000 in PBS with 0.5% FBS was added (100 μL per well) and incubated for 1 h at room temperature. After washing as above, filtered BCIP/NBT (bromo-chloro-indolyl phosphate/nitroblue tetrazolium)-Plus substrate (Mabtech) was added (100 μL per well) and after 25-min development, the plates were rinsed with tap water and left to dry. The spots (representing imprints of cytokine-producing cells) on the membranes were counted by an automated ELISpot reader [Autoimmune Diagnostika (AID) GmbH, Strassberg, Germany] using the reader's software (AID ELISpot reader 3.5). The results were expressed as the mean of spots/number of cells plated. For IL-4, IL-5 and IL-13, an individual's response to parthenolide was considered positive if it was more than two times higher than the value of unstimulated PBMC and displayed a minimum value of > 5.0 spots per 300 000 PBMC after subtraction of spontaneous spots. For IL-2 and IFN- γ , because of a higher spontaneous background in comparison with the other cytokines, an increase > 15 spots per 300 000 PBMC was used as a single cut-off criterion.

Depletion of CD4+ or CD8+ cells

PBMC from two subjects with parthenolide allergy and one subject with nickel allergy were depleted of CD4+ or CD8+ cells. The depletion procedure using BD IMag™ antihuman

CD4 or CD8 magnetic nanoparticles – DM (BD Biosciences, San Jose, CA U.S.A.) was performed in accordance with the manufacturer's instructions. Briefly, PBMC were washed with BD IMag™ buffer and incubated with antihuman CD4 or CD8 mAb-conjugated nanoparticles. The PBMC were diluted with BD IMag™ buffer and incubated with a BD IMagnet to remove CD4+ or CD8+ cells. The CD4+ and CD8+-depleted cell fractions were washed in cell medium and finally diluted to 3.0×10^6 cells mL^{-1} . Cytokine production was measured by ELISpot using PBMC and depleted CD4+ and CD8+ cell fractions with or without 1.1 $\mu\text{mol L}^{-1}$ parthenolide or 50 $\mu\text{mol L}^{-1}$ $\text{NiCl}_2 \times 6 \text{H}_2\text{O}$ (Sigma) according to the protocol above. The grade of depletion was analysed with a FACScan (BD Bioscience) using the software Cellquest (BD Bioscience). Fluorescein isothiocyanate-conjugated anti-CD4 or -CD8 mAbs (Mabtech) were used according to the manufacturer's instructions. The FACScan was gated within the lymphocyte population. For the two parthenolide-reactive PBMC, the depletion of CD4+ cells was 96.5% and 99.8%, and for CD8+ cells 93.6% and 98.3%. Corresponding values for the nickel-reactive PBMC fractions were 98.9% and 80.7%.

Statistical analysis

Statistical analyses were performed using Analyse-it for Microsoft Excel version 1.65 (Analyse-it Software Ltd, Leeds, U.K.). The Mann-Whitney U-test (one-sided) was used to compare the responses of the allergic and the control groups. The correlation between the production of different cytokines was analysed using Spearman's rank order correlation coefficient, r_s . The background values (medium without parthenolide) were subtracted from the parthenolide-induced values prior to statistical analyses. If negative values were obtained, the values were set to 0.

Results

Defining a test protocol for assessment of cytokine responses to parthenolide

Prior to the analyses of the cytokine profile induced by parthenolide in PBMC from the study groups, the optimal concentrations of parthenolide for induction of hapten-specific cytokine production were defined in pilot tests. PBMC from patch test-positive and -negative subjects were stimulated with parthenolide at 0.041–50 $\mu\text{mol L}^{-1}$ and the production of IFN- γ , IL-4 and IL-13 was measured. Optimal cytokine responses in PBMC from allergic subjects, but not control subjects, were obtained at concentrations ranging from 0.37 to 3.3 $\mu\text{mol L}^{-1}$ as exemplified by IL-13 ELISpot results in Figure 1. The reduction of responses when exceeding 3.3 $\mu\text{mol L}^{-1}$ was probably due to toxicity as suggested by separate experiments demonstrating a clear inhibition of mitogen (PHA)-induced cytokine responses at parthenolide concentrations exceeding 4.0 $\mu\text{mol L}^{-1}$ (data not shown).

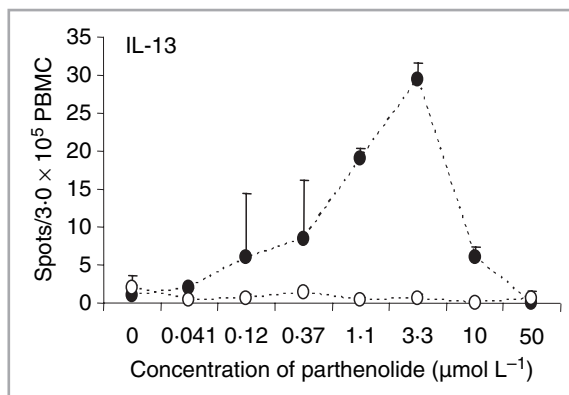


Fig 1. Optimal concentrations of parthenolide required for induction of *in vitro* cytokine responses in peripheral blood mononuclear cells (PBMC) from subjects with allergic contact dermatitis (ACD) to parthenolide. Interleukin (IL)-13 production by PBMC from allergic (●, $n = 2$) and nonallergic (○, $n = 3$) subjects were analysed in triplicate (3.0×10^5 cells per well) in the presence or absence of parthenolide by enzyme-linked immunospot (ELISpot) assay. Parthenolide at concentrations of 0.37, 1.1 and $3.3 \mu\text{mol L}^{-1}$ induced the highest IL-13 responses in allergic subjects. The mean and SD for the response of the allergic and control subjects are shown.

Increased cytokine production induced by parthenolide in peripheral blood mononuclear cells from subjects with contact allergy

The ability of parthenolide, at concentrations of 0.37, 1.1 and $3.3 \mu\text{mol L}^{-1}$, to stimulate the production of cytokines by PBMC from subjects with ($n = 10$) or without ($n = 5$)

patch-test reactivity to parthenolide was measured by ELISpot.

As seen in Figure 2, parthenolide induced an increased number of cells producing IFN- γ , IL-2, IL-4, IL-5 and IL-13, in the allergic but not in the control group. The difference between the groups was statistically significant for all cytokines, albeit not always at all concentrations of parthenolide. For IL-5 and IL-13, all three concentrations of parthenolide elicited significant responses in the allergic group whereas the significance of the IFN- γ , IL-2 and IL-4 response was limited to one or two concentrations (Fig. 2). The number of cells producing the different cytokines in PBMC from the allergic group following stimulation with the hapten correlated with each other at all comparisons performed, i.e. using data from any of the three concentrations of parthenolide. For example, when comparing the responses to parthenolide at $1.1 \mu\text{mol L}^{-1}$ the production of the T-cell type 2 cytokine IL-13 correlated with the production of both the T-cell type 1 cytokines IFN- γ ($r_s = 0.88$, $P < 0.001$) and IL-2 ($r_s = 0.81$, $P < 0.01$) and the T-cell type 2 cytokines IL-4 ($r_s = 0.89$, $P < 0.001$) and IL-5 ($r_s = 0.97$, $P < 0.001$).

A summary of the results obtained at an individual level is shown in Table 1. Based on cut-off definitions, all eight individuals with moderate or strong patch-test reactions responded to parthenolide by production of IFN- γ . Seven of these were also positive for IL-13 together with IL-2, IL-4 and/or IL-5. In five subjects, all five cytokines were detected. The controls and the two subjects with weak patch-test reactions did not respond. As seen in Figure 3, the magnitude of IL-13 and IFN- γ responses, the two cytokines with which most subjects responded, varied between subjects.

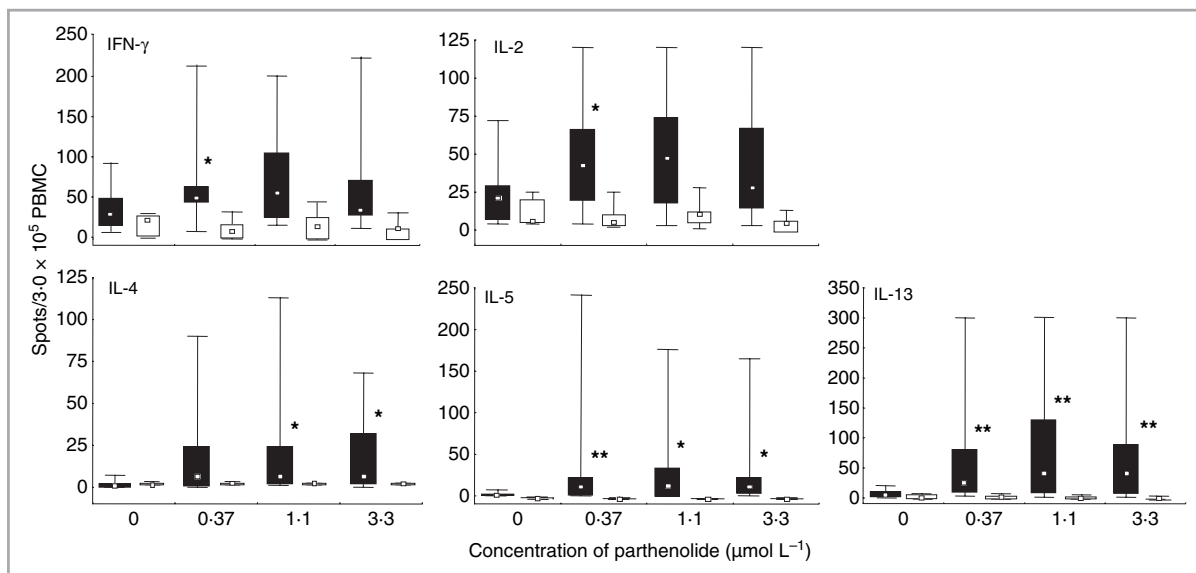


Fig 2. Induction of T-cell type 1 [interferon (IFN)- γ and interleukin (IL)-2] and type 2 (IL-4, IL-5 and IL-13) cytokine responses to parthenolide. Peripheral blood mononuclear cells (PBMC) from parthenolide-allergic ($n = 10$; $n = 9$ for IL-5; filled boxes) and nonallergic subjects ($n = 5$; $n = 4$ for IL-5; open boxes) were tested in triplicate (3.0×10^5 cells per well) in the presence or absence of parthenolide by enzyme-linked immunospot (ELISpot) assay. The box plots show the median ($\square$), min ($\perp$) and max ($\top$), and 25th and 75th percentiles ($\square$) for the respective group at each concentration of parthenolide (* $P < 0.05$, ** $P < 0.01$).

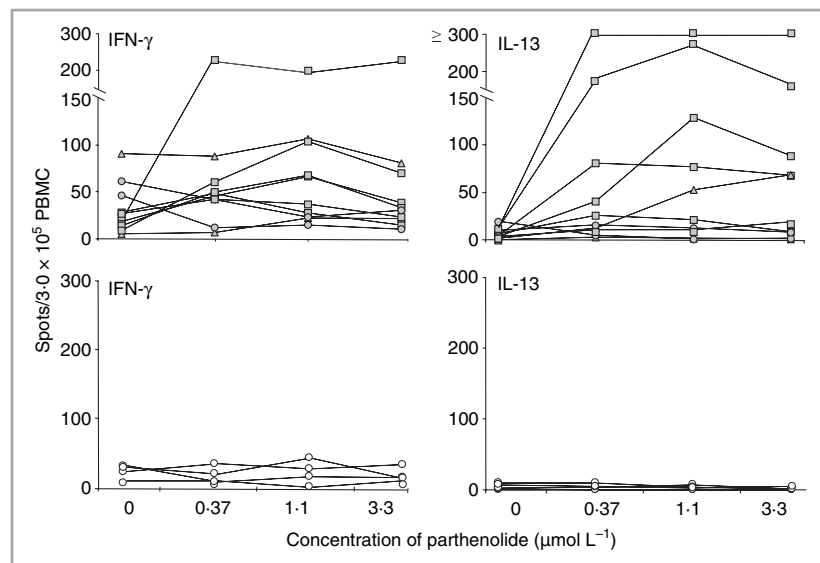
Table 1 Cytokine response to parthenolide in peripheral blood mononuclear cells from subjects with or without allergic contact dermatitis

Subject	Patch-test reactivity to parthenolide	T-cell type 1 cytokines		T-cell type 2 cytokines		
		IFN- γ	IL-2	IL-4	IL-5	IL-13
Allergic						
1	+++	●	●	●	●	●
2	+++	▲	○	○	●	●
3	+++	●	●	●	●	●
4	+++	●	●	●	●	●
5	+++	▲	○	●	○	●
6	+++	●	●	●	●	●
7	++	▲	○	○	n.d.	○
8	++	●	▲	●	●	●
9	+	○	○	○	○	○
10	+	○	○	○	○	○
Controls						
11	—	○	○	○	○	○
12	—	○	○	○	○	○
13	—	○	○	○	n.d.	○
14	—	○	○	○	○	○
15	—	○	○	○	○	○

The circles show the individual cytokine responses to three concentrations of parthenolide: 0.37, 1.1 and 3.3 $\mu\text{mol L}^{-1}$. (●) Value from two or three concentrations above cut-off; (▲) value from one concentration above cut-off; (○) values below cut-off. The cut-off for positive responses was defined as described in Material and methods.

IFN, interferon; IL, interleukin; n.d., no data.

Fig 3. Parthenolide-induced interferon (IFN)- γ and interleukin (IL)-13 responses by peripheral blood mononuclear cells (PBMC) from individual subjects with or without allergic contact dermatitis (ACD) to parthenolide. PBMC from subjects with ACD to parthenolide (■, +++; ●, ++ and ▲, + patch-test reaction; upper graphs) or control subjects (○, negative patch test; lower graphs) were tested in triplicate (3.0×10^5 cells per well) in the presence or absence of parthenolide by enzyme-linked immunospot (ELISpot) assay.



Parthenolide induces CD8+ T cell-mediated production of interferon- γ and interleukin 13

To determine the phenotype of the cells responsible for the cytokine production, parthenolide-induced production of IFN- γ and IL-13 was further studied in PBMC from two allergic subjects after depletion of either CD4+ or CD8+ cells (Fig. 4). As seen in the figure, both the whole population of PBMC and PBMC depleted of CD4+ cells displayed IL-13

responses to parthenolide. In contrast, PBMC depleted of CD8+ cells were almost completely devoid of reactivity, showing that CD8+ T cells, but not CD4+ cells, were required for the response. Similar results were obtained for IFN- γ production. Completely opposite results were obtained for the IL-13 response of nickel-stimulated PBMC from a nickel-allergic subject included as an internal control in the experiments. The production of IL-13 by CD8+-depleted cells and not by CD4+-depleted cells was confirmed in five additional

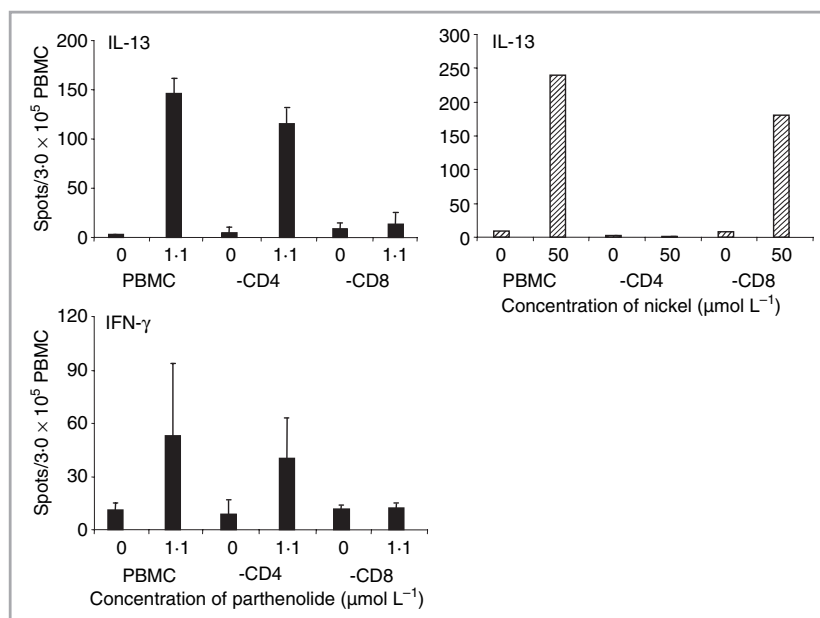


Fig 4. Phenotypic characterization of parthenolide-specific cells producing interleukin (IL)-13 and interferon (IFN)- γ . Peripheral blood mononuclear cell (PBMC) suspensions from two subjects with allergic contact dermatitis to parthenolide (left graphs) were depleted of CD4+ or CD8+ cells. The PBMC and the depleted cell fractions were tested in triplicate (3.0×10^5 cells per well) in the presence or absence of parthenolide and the cytokine-producing cells were measured by enzyme-linked immunospot (ELISpot) assay. The data were obtained from one of two experiments with reproducible results and are displayed as the mean response + SD of the subjects. For comparison, PBMC from a subject with allergy to nickel (right graph) were analysed by the same protocol using nickel for induction of IL-13-producing cells.

nickel-reactive subjects (data not shown). This pattern of reactivity to nickel is in line with previous observations that nickel-specific responses manifested by, for example, IL-4 and IFN- γ production are primarily attributable to CD4+ T cells.^{5,10}

Discussion

In this study, the *in vitro* cytokine profile and the phenotype of the cytokine-producing cells induced by the sesquiterpene lactone parthenolide were assessed using PBMC from human subjects with contact allergy to parthenolide. The analyses revealed an *in vitro* response involving concomitant and correlated production of IFN- γ , IL-2, IL-4, IL-5 and IL-13 with a significantly higher response in PBMC from allergic compared with nonallergic subjects. Whereas IFN- γ and IL-2 represent a T-cell type 1 response, IL-4, IL-5 and IL-13 represent type 2 cytokines; IL-2 is in addition produced by T cells prior to their commitment into T-cell type 1 or 2 cells and is, in humans, less restricted to T-cell type 1 clones than IFN- γ .²⁴ The T cells responsible for the production of IFN- γ and IL-13 were found to be of the CD8+ cell subset.

Although parthenolide elicited a cytokine pattern similar to that previously described for nickel-reactive PBMC, the T cells responsible for the cytokine response to the respective haptens differed. The involvement of CD8+ T cells in the peripheral response to parthenolide, albeit demonstrated in a limited number of subjects, is in strong contrast to the CD4+-mediated nickel-induced production of T-cell type 1 and 2 cytokines previously shown and confirmed with regard to IL-13 in this study.^{5,10} This difference suggests that the chemical properties of a hapten have an impact on the resulting immune response. Parthenolide and nickel display quite different features, one hapten being an organic molecule and the other a metal ion. They thus differ in how they interact with, and

bind to, proteins¹ but also with regard to their solubility properties; nickel ions display high solubility in water whereas parthenolide is a lipophilic compound.

Notably, solubility properties could influence the ability of a hapten to penetrate the skin, its bioavailability and also its capacity to reach various compartments of antigen-presenting cells (APCs).¹² In contrast to hydrophilic compounds, lipophilic compounds may display a better capacity to pass through membranes of the APC and hence access the cytosol, which may favour presentation on major histocompatibility complex class I molecules similarly to antigens presented by the endogenous pathway.^{25,26} This would favour recognition by CD8+ T cells, in line with the observations made in this study. It is, however, important to note that although parthenolide-reactive CD8+ T cells secrete IFN- γ and IL-13, concomitant involvement of CD4+ T cells producing other cytokines cannot be ruled out.

The knowledge on the immunological response of human PBMC reactive with organic haptens, in terms of cytokines produced and the T-cell phenotypes involved, is limited. It is thus difficult to draw general conclusions about the impact of the properties of an organic hapten on the resulting immunological mechanisms. With regard to the cytokine profile, mixed T-cell type 1 and 2 cytokine responses have previously been described for PBMC reactive with lipophilic haptens in the fragrance mix from the European patch test standard series⁴ as well for PBMC reactive with the hydrophilic hapten mix MCI/MI.¹¹ In neither of these studies were the cells responsible for the cytokine production defined. Studies on what cell phenotypes are involved in the response to organic haptens in human ACD are instead based on the generation and analysis of T-cell clones (TCC). In the case of TCC to components of the fragrance mix, the TCC generated were predominantly CD4+ T cells that displayed T-cell type 1, but not type 2, cytokine production.²⁷ In contrast, generation of

TCC to the lipophilic hapten urushiol yielded CD8+ TCC secreting type 1 cytokines.²⁸ It is, however, important to be cautious when comparing results obtained with PBMC and TCC. Whereas analysis of PBMC will provide a general overview of the predominating *ex vivo* response of peripheral bulk cells, TCC represent single cells capable of surviving long-term culture and repeated stimulation *in vitro*. Also, the generation of TCC can have an impact on cell differentiation and the secretion of cytokines and other factors.^{29,30} An example of differential results obtained with PBMC vs. TCC is found in the analyses of reactivity to nickel. Nickel-stimulated PBMC from nickel-allergic individuals have been shown to display a predominant CD4+ T cell-mediated cytokine production^{5,10} whereas human TCC generated to nickel have been of both the CD4+ and CD8+ subset.^{31,32} Nevertheless, the discrepancies observed herein between parthenolide and nickel, in terms of the involvement of CD4+ vs. CD8+ T cells, emphasize that the character of the peripheral immune response to contact allergens in humans cannot be described by one single model. Another relevant issue is how the differences in the peripheral T-cell response to haptens translate to the T-cell reactivity displayed in the skin. This is not known in the case of parthenolide, and for nickel, contradictory reports have described TCC from skin infiltrates comprising predominantly CD4+ T cells, mixed CD4+/CD8+ T cells or predominantly CD8+ T cells.^{31,33,34}

Not only may the nature of haptens have an impact on which T-cell subsets are activated, but also the activation of APC, cells of vital importance for the divergent activation of CD4+ vs. CD8+ T cells, may depend on the nature of the haptens. In a recent study by Toebak *et al.*, it was shown that organic compounds defined as contact allergens [2,4-dinitrochlorobenzene (DNCB) and oxazolone] skewed the differentiation of dendritic cells (DC) into DC type 1 (DC1) whereas trimellitic anhydride (TMA), defined as a chemical respiratory allergen, promoted polarization of DC to the DC2 phenotype.³⁵ The differential activation of DC by these compounds may, irrespective of whether they are contact sensitizers or respiratory allergens, relate to their chemical nature; both DNCB and oxazolone display solubility properties similar to parthenolide whereas TMA is hydrophilic. Nickel, investigated in the same study, yielded a mixed DC1 and DC2 activation.

Attempts to investigate immune responses of PBMC potentially reactive with various haptens may be hampered by technical obstacles including the aspect of adding a highly lipophilic hapten to cell cultures in a water-based medium. This problem has been addressed previously when analysing cellular reactivity to fragrances.²⁷ Notably, the results of the present study demonstrate that a lipophilic hapten, requiring presolubilization in an organic solvent, can be added to PBMC and induce a detectable immune response. This should facilitate further studies on other organic haptens presenting similar solubility issues.

The possibility of detecting immunological reactivity to parthenolide by short-term *ex vivo* stimulation of PBMC may also

be of relevance for the future development of *in vitro*-based diagnostic methods. This has previously been proposed for nickel but may also be applicable for organic compounds as indicated by this study and other studies on additional metal salts, fragrances and MCI/MI.^{2,3,11,36} Limitations in the detection sensitivity displayed by *in vitro* methods, possibly suggested by the fact that two subjects with weak patch-test reactivity to parthenolide did not respond *in vitro*, could potentially be overcome by employing strategies to amplify the *in vitro* reaction, e.g. by stimulating PBMC with haptens in the presence of stimulatory cytokine cocktails.^{4,7} Although the patch test is well established and routinely used for diagnosis of contact allergy and ACD, the method has some drawbacks such as severe reactions, false-negative, false-positive reactions, subjectivity by the examiner, inconveniences for the patient, etc.^{37,38} In addition, with regard to sesquiterpene lactones, including parthenolide, due to their strong sensitizing capacity, there is a risk of active sensitization when plant extracts or *Compositae* mix are used for patch testing.^{20,39}

In conclusion, the present study demonstrates that immunological responses of PBMC to a lipophilic hapten can be assessed by *in vitro* methods previously used in studies on water-soluble substances. In comparison with nickel allergy, the most studied type of contact allergy in humans, the cytokine response to parthenolide was manifested by a similar mixed T-cell type 1 and 2 cytokine profile but differed with regard to the phenotype of the T cells responsible for the cytokine production.

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