

Nickel, cobalt, chromium, palladium and gold induce a mixed Th1- and Th2-type cytokine response *in vitro* in subjects with contact allergy to the respective metals

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Summary

Nickel (Ni), the main cause of contact allergy to metals, induces *in vitro* production of both Th1- and Th2-type cytokines in peripheral blood mononuclear cells (PBMC) from allergic subjects. Because the knowledge of the cellular immune response to other metals involved in contact allergy has been limited, we investigated the cytokine profile induced by Ni, cobalt (Co), chromium (Cr), palladium (Pd) and gold (Au) in PBMC from patients with patch test reactivity to the respective metals. PBMC from patients with patch test reactivity to Ni, Co, Cr, Au and/or Pd ($n = 31$) and non-allergic controls ($n = 5$) were stimulated *in vitro* with corresponding metal salts. Th1- [interleukin (IL)-2 and interferon (IFN)- γ] and Th2- (IL-4 and IL-13) type cytokine responses were measured by enzyme-linked immunospot (ELISpot) and/or enzyme-linked immunosorbent assay (ELISA). All metals induced a mixed Th1- and Th2-type cytokine production in PBMC from individual patients with patch test reactivity to the corresponding metal, but not in control PBMC. Significantly higher responses in the patient *versus* controls were found for Cr (IL-2 and IL-13), Pd (IL-2 and IL-4), Au (IL-13 and IFN- γ) (all $P < 0.05$) and Ni (all four cytokines; $P < 0.01$) but not Co. Overall, 71% (37/52) and 89% (81/91) of the positive and negative patch test reactivities to metals, respectively, were matched by the *in vitro* reactivity. In conclusion, our data suggest that sensitization to Co, Cr, Pd and Au results in a cellular immune response of a character similar to the mixed Th1- and Th2-type cytokine profile shown previously to be induced by Ni.

Keywords: allergic contact dermatitis, cytokines, ELISA/ELISpot

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Introduction

Sensitization to nickel (Ni) is the main cause of metal-induced allergic contact dermatitis (ACD) [1,2]. However, other metals including cobalt (Co), chromium (Cr), palladium (Pd) and gold (Au), are also known to cause ACD [3,4]. Recent attempts to diminish Ni use in products placed in direct and prolonged contact with the skin [5,6] have led to an increase in the use of other metals in alloys used in dentistry, jewellery and orthopaedics. This, in turn, has led to a rise in the incidence of ACD reactions subsequent to exposure to other metal contactants [3,4]. Unlike ACD to Ni, where the *in vitro* cytokine responses by peripheral blood mononuclear cells (PBMC) or specific T cell clones have been described extensively [7–12], few studies have addressed the immunological cytokine profile induced *in vitro* by other metal sensitizers.

The standard series of haptens used for patch testing commonly includes, among 25–30 of the most prevalent haptens, the metal ions Ni, Co and Cr [13]. Patch test series used to investigate possible ACD reactions associated with, e.g. dentistry or dental applications often includes the additional metals Au, Pd and mercury (Hg) [14,15]. In subjects with metal ACD, e.g. jewellery reactors, reactivity with Ni is most prevalent followed by reactivity with Co, Pd, Au and Cr (e.g. 94.5%, 34%, 17%, 10% and 3%, respectively [14]). It is common that the patients are sensitized to multiple metal ions [14,16,17]. Whether this is due to concurrent sensitization, cross-reactivity or, in some cases, both is not fully understood [18–20]. However, several studies have suggested that sensitization to one metal ion increases the chance of being sensitized to additional metals [4,21,22].

The response to Ni *in vitro* has been shown to involve activation of Ni-specific T cells followed by proliferation and

induction of both Th1- [e.g. interleukin (IL)-2 and interferon (IFN)- γ], Th2-type (e.g. IL-4, IL-5 and IL-13) as well as regulatory (e.g. IL-10) cytokines [10–12,23–25]. With regard to metal contact allergens other than Ni, there have been reports of elevated proliferative responses in the lymphocyte transformation test (LTT) and expression of mRNA encoding for a number of proinflammatory cytokines [e.g. tumour necrosis factor (TNF)- α , IL-6 or IL-1 α] by PBMC from subjects with metal restorations, orthopaedic implants [26–28] or rheumatoid arthritis [29–31]. However, while these studies show that these metals are capable of inducing cellular activation *in vitro*, more studies are needed that specifically address functional aspects of the immune response in terms of the profile of Th1- and Th2-type cytokines elicited by each metal.

The aim of the present study was therefore to determine the *in vitro* cytokine responses in PBMC from subjects with patch test reactivity to different metals including Ni, Co, Cr, Pd and Au. The findings suggest that all these metals elicit a similar response in allergic subjects involving both Th1- and Th2-type cytokine production.

Materials and methods

Patients and patch tests

The study included 31 patients with a history of eczematous reactions typical of ACD and a positive patch test to metals and five healthy volunteers with no history of ACD and a negative patch test (Table 1). The subjects were patients or staff of the Karolinska University Hospital, Stockholm, Sweden. Patch test reactivity was established 1 week–48 months before blood samples for this study were taken. The patch test was performed by applying FinnChambers (Epitest Ltd Oy, Tuusula, Finland) with a series of haptens for 48 h on the backs of the subjects and were scored at days 3 and 7, by two independent experienced personnel, using the International Contact Dermatitis Research Group (ICDRG) scoring system [1]. Patients referred to patch testing due to eczematous skin reactions were tested with a standard patch test series including NiSO₄ (5%), K₂Cr₂O₇ (0.5%) and CoCl₂ (1%), whereas patients suffering from oral reactions as well as controls were patch tested with a dental series also including PdCl₂ (1%) and Na₃[Au(S₂O₃)₂] (0.5%; all salts applied in petrolatum). Patch test reactivity was defined as strong (+3; oedema, erythema, papules and vesicles), moderate (+2; oedema, erythema and papules), weak (+1; oedema and erythema) or no reaction (0). The study was approved by the Ethics Committee at the Karolinska University Hospital, Stockholm, Sweden (ethical permission no: 00–238). All subjects gave their informed consent to participation.

Metal salts used for *in vitro* cytokine assays

The metal salts used for *in vitro* tests were nickel chloride (NiCl₂), nickel sulphate (NiSO₄), chromium chloride [CrCl₃];

Cr(III)], potassium dichromate [K₂Cr₂O₇; Cr(VI)], cobalt chloride (CoCl₂), palladium chloride (PdCl₂), gold chloride [HAuCl₄; Au(III)] (all from Sigma-Aldrich, St Louis, MO, USA) and gold sodium thiosulphate {Na₃[Au(S₂O₃)₂]; Au(I)}; (MP Biomedicals, LCC, Irvine, CA, USA).

Blood sample collection and processing

Blood samples were obtained by venepuncture and in sterile heparinized glass vials. The PBMC were separated by density-gradient centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden) and frozen as described [12]. Prior to testing, PBMC were thawed at 37°C and washed twice with medium [RPMI-1640 containing 20 mM HEPES buffer supplemented with L-glutamine (2 mmol/l), penicillin G sodium (100 units/ml), streptomycin sulphate (100 µg/ml)] containing 10% heat-inactivated fetal bovine serum (FBS). The viability of cells was confirmed by trypan blue exclusion (> 90% viable cells).

Cytokine enzyme-linked immunospot assay (ELISpot)

ELISpot was performed essentially as described [32]. Briefly, polyvinyl difluoride plates (ELIIP10SSP; Millipore Corporation, Bedford, MA, USA) were coated with 100 µl of capture monoclonal antibodies (mAbs) at 15 µg/ml in sterile phosphate-buffered saline (PBS) per well overnight at +4°C. The mAbs used for capture were IL2-I (IL-2), 82.4 (IL-4) and IL-13-I (IL-13) (all from Mabtech, Stockholm, Sweden). Prior to use, the wells were blocked with 200 µl of complete medium containing 10% heat-inactivated FBS at room temperature (RT) for 1 h. PBMC suspensions (100 µl) with or without addition of stimuli were added to the wells and following the incubation, cytokine production by PBMC was detected by incubating the plates with biotinylated secondary mAbs (1 µg/ml in PBS/0.5% FBS); IL2-II (IL-2), 12.1 (IL-4) and IL13-II (IL-13) (Mabtech) for 2 h at RT and subsequently with a streptavidin–alkaline phosphatase conjugate (SA-ALP) (50 ng/ml) (Mabtech). Plate incubation with substrate (bromo-chloro-indolylphosphate/nitroblue tetrazolium; Mabtech) and enumeration of spots representing cytokine-producing cells was performed as described previously [12].

Cytokine enzyme-linked immunosorbent assay (ELISA)

The cytokine levels in PBMC supernatants obtained as described below were measured by ELISA as described previously [12]. MAb IL2-I (IL-2), IL13-I (IL-13) and 1-D1K (IFN- γ) were used for capture and biotinylated mAb IL-2-II (IL-2), IL13-II (IL-13) and 7-B6-1 (IFN- γ) were used for detection (Mabtech); standard curves were obtained using recombinant human IL-2, IL-13 or IFN- γ (Mabtech). The lowest detection limit of the assays was determined to

Table 1. Subjects and patch test reactivity to metals.

Subject no.	Sex (F/M)	Age (years)	Ni	Co	Cr	Pd	Au	Time ^b (months)
ACD ^a (standard series; <i>n</i> = 16)								
1	F	78	+3	+3	+1			11
2	F	55	–	–	+3			7
4	F	54	–	–	+3			1
6	F	20	+3	+2	–			20
7	F	53	+3	+2	–			20
8	F	29	+2	+2	–			24
9	F	52	+3	+2	–			7
11	F	34	–	+2	–			14
15	F	60	–	–	+3			38
20	M	53	+1	+2	+3			7
21	F	21	–	+2	–			9
22	F	28	–	–	+2			12
24	F	50	–	+2	+2			9
27	F	56	+2	–	–			2
28	F	41	+3	–	–			11
29	F	60	+3	–	–			0.25
ACD (dental series; <i>n</i> = 15)								
3	F	69	–	–	–	–	+2	19
5	F	50	+3	+3	–	–	–	14
10	F	65	–	–	–	–	+2	14
12	M	63	–	–	–	–	+3	11
13	F	58	+3	–	–	+1	+3	48
14	F	66	–	–	–	–	+2	19
16	F	62	+3	+1	–	+2	–	36
17	M	45	–	–	–	–	+2	17
18	F	55	+3	–	+1	–	–	16
19	F	72	+3	–	+1	+1	+1	30
23	F	73	+2	–	–	–	+1	8
25	F	54	–	+1	–	+2	+2	30
26	F	50	–	–	–	–	+3	1
30	F	60	+3	–	–	–	–	7
31	F	55	+2	–	–	–	–	10
Controls								
1	F	66	–	–	–	–	–	11
2	F	48	–	–	–	–	–	9
3	F	51	–	–	–	–	–	9
4	F	68	–	–	–	–	–	9
5	M	74	–	–	–	–	–	4

Patients tested with the standard series were tested for Ni (NiSO₄), Co (CoCl₂) and Cr (K₂Cr₂O₇); Patients tested with the dental series and control subjects were tested also with Pd (PdCl₂) and Au [Na₃(Au(S₂O₃)₂)]. ^aSubjects with *in vivo* patch test reactivity to one or several metals included in the standard or dental test series; ^btime (months) between patch test and blood sample.

5 pg/ml as described previously [12]. For all calculations and statistical analyses, values < 5 pg/ml were therefore defined as 5 pg/ml.

PBMC stimulations to define optimal conditions for *in vitro* cytokine assays

Toxicity of metal salt

To assess toxicity, defined as the capacity of a metal salt to

down-regulate mitogen-induced cytokine production, 1.0×10^5 PBMC/well from non-allergic donors were stimulated with 2 µg/ml of phytohaemagglutinin (PHA; Orion Diagnostics, Trosa, Sweden) in the presence or absence of titrations of the different metal salts. PBMC were incubated at +37°C and 5% CO₂ in humidified air and cytokine production was measured by ELISpot after 20 h (IL-2) or 40 h (IL-4 and IL-13). The metal salt concentration required to reduce the PHA-induced cytokine production by 25% was used to define the toxicity.

Definition of optimal metal salt concentrations for antigen-specific cytokine production

To determine the optimal concentration of each metal salt to be used for specific induction of cytokine responses, PBMC (3.0×10^6 cells/ml) from selected patients with strong patch test reactivity were incubated in the absence or presence of a concentration range of the metal to which the patient reacted in the patch test. Control PBMC were analysed in parallel. Cytokine ELISpot assays were set up in triplicate with 100 μ l of PBMC suspension per well. Incubation times at +37°C and 5% CO₂ in humidified air were 20 h for IL-2 and 40 h for IL-4 and IL-13 assays.

Analysis of cytokine production in response to a panel of metal salts

PBMC (3.0×10^6 cells/ml) were incubated at +37°C and 5% CO₂ in humidified air with or without all different metal salts at optimal concentrations defined for each metal salt as above. As a cell viability control, 1.0×10^5 PBMC with PHA (2 μ g/ml) was included. For ELISpot, triplicates of 100 μ l/well of PBMC suspension were incubated for 20 h (IL-2) or 40 h (IL-4 and IL-13). For IL-2, IL-13 and IFN- γ ELISA, 2 ml PBMC suspensions were incubated for 40 h. The supernatants were recovered by centrifugation and stored frozen at -20°C prior to analysis.

Statistical analysis

The Mann-Whitney *U*-test was used to compare the cytokine responses between ACD and control subjects. For comparison of metal-specific cytokine responses, values with background (spontaneous production) subtracted were used. A *P*-value < 0.05 was considered to be statistically significant. All tests were performed using the software STATISTICA version 5.1 (StatSoft, Tulsa, OK, USA).

Cut-off definitions for positive *in vitro* responses to a panel of metals in cytokine assays

The cut-off for positive responses in ELISpot and ELISA was defined by two criteria, cytokine increment (metal-induced minus spontaneous production) and ratio (metal-induced response divided by the spontaneous production). A response was considered positive when both the increment and ratio were higher than the cut-off values. Two spots were added to all ELISpot values to be able to calculate ratios; ELISA values < 5 pg/ml were set to 5 pg/ml. Cut-off values were set to be well above the increment and ratio values obtained with metal-stimulated control PBMC. Because Ni and Pd stimulation resulted in some elevation of some cytokines, in particular IL-2 and also in the controls, cut-off values were set differentially depending on cytokine assay

and the metal used for stimulation (Ni or Pd as opposed to Au, Co or Cr). The following cut-off values were used; IL-2 ELISA; for Ni/Pd, increment ≥ 80 pg/ml and ratio $\geq 16\times$; for Au/Co/Cr, increment ≥ 20 pg/ml and ratio $\geq 4\times$; IL-13 ELISA: for all metals, increment ≥ 12.5 pg/ml and a ratio of $\geq 4\times$ or $3.3\times$ for Ni/Pd and Au/Co/Cr, respectively; IFN- γ ELISA, for all metals increment ≥ 30 pg/ml and a ratio of $\geq 4.5\times$ or $4\times$ for Ni/Pd and Au/Co/Cr, respectively; IL-2 ELISpot; for Ni/Pd, increment ≥ 15 spots and ratio $\geq 3\times$; for Au/Co/Cr, increment ≥ 12 spots and ratio $\geq 2\times$; IL-4 and IL-13 ELISpot; for all metals, increment ≥ 4 or 10 spots, respectively, and ratio $\geq 2\times$.

Results

Anamnestic data on patients and controls

Patients were selected on the basis of +3 (strong) or +2 (moderate) patch test reactivity to Ni, Co, Cr(VI), Pd and/or Au(I), whereas the controls were negative (Table 1). Sixteen patients were tested with a standard series (Ni, Co and Cr) while the remaining patients and the controls were also patch tested with Au and Pd. Many patients displayed reactivity with multiple metals, most commonly between Ni and Co, and in some cases additional patch test positive reactions were +1 (weak).

Definition of optimal conditions for measurement of metal-induced cytokine responses

The toxicity of the metals, defined by a reduction of the PHA-induced response, was assessed by measurement of IL-2, IL-4 and IL-13 production by ELISpot using PBMC from non-allergic subjects. The toxicity ranged from 1.9 μ M for Cr(VI) to > 500 μ M for Cr(III) (Table 2). The optimal concentration for induction of metal-specific responses in PBMC from selected patients with strong patch test reactivity, but not in controls, was defined by pilot studies using IL-2, IL-4 and IL-13 ELISpot and ranged from 0.5 μ M for Cr(VI) to 100 μ M for Cr(III) and PdCl₂ (Table 2). Cr(III) yielded higher cytokine responses in patients despite the preferred use of Cr(VI) in the patch test [14,18]. However, Cr(III), similar to Cr(VI), is capable of eliciting eczema at low concentrations [15]. Au(I) elicited higher responses than Au(III) *in vitro* in PBMC from patients, in line with what has been observed in the patch test [33]. For Ni, NiCl₂ and NiSO₄ yielded comparable results in all tests. For the following analyses of metal-induced cytokine responses, only responses to NiCl₂, CoCl₂, Cr(III), PdCl₂ and Au(I) are shown.

Co, Cr, Pd and Au elicit a cytokine profile similar to that elicited by Ni in PBMC from metal-allergic patients but not control subjects

PBMC from all patients and controls (Table 1) were analysed for reactivity to a panel of metals at optimal concentrations

Table 2. Metal salt toxicity and optimal concentration for specific stimulation of cytokine responses.

Metal (oxidation state)	Metal salt	Range tested ^a (μM)	Toxicity ^b (μM)	Optimal dose ^c (μM)
Ni (II)	NiCl ₂	2–500	330	50
Ni (II)	NiSO ₄	2–500	300	50
Co (II)	CoCl ₂	2–500	300	50
Cr (III)	CrCl ₃	2–500	> 500	100
Cr (VI)	K ₂ Cr ₂ O ₇	0.05–50	1.9	0.5
Pd (II)	PdCl ₂	2–250	> 250	100
Au (I)	Na ₃ [Au(S ₂ O ₃) ₂]	1–250	50	25
Au (III)	HAuCl ₄	0.1–500	70	2

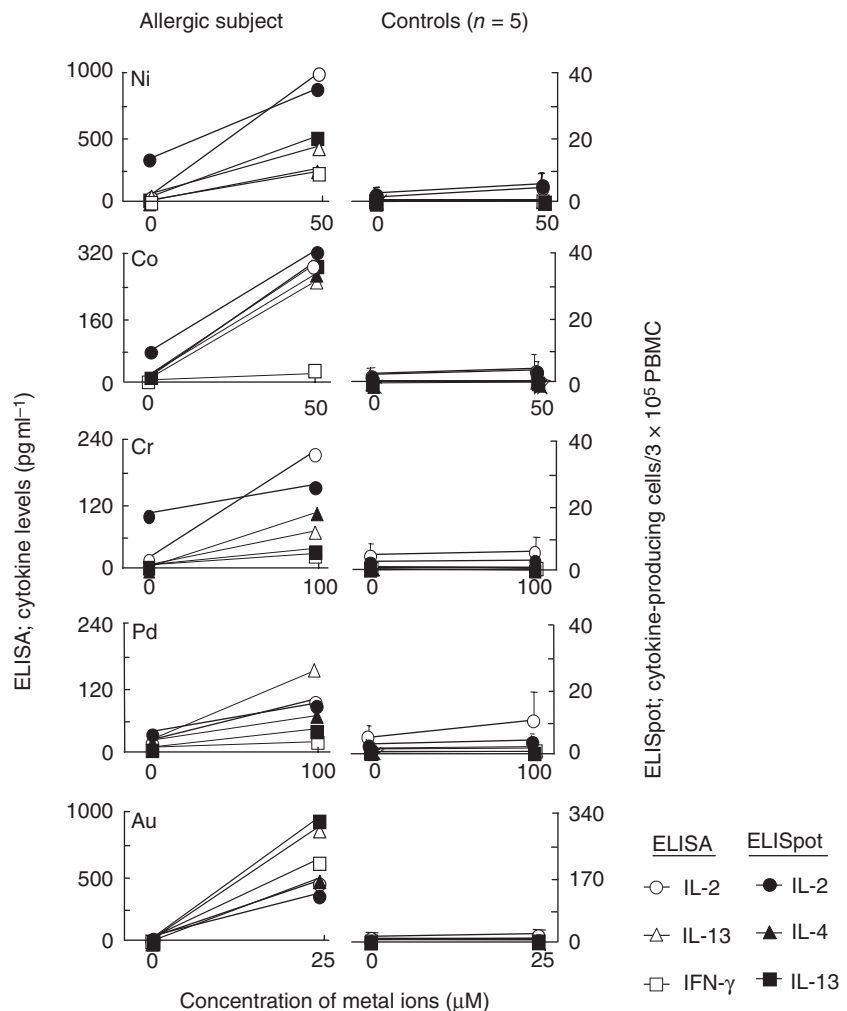
^aDifferent concentrations were used for each salt depending on the solubility and toxicity; ^btoxicity was defined by the average concentration of metal salt required to reduce the phytohaemagglutinin (PHA)-induced interleukin (IL)-2, IL-4 and IL-13 production to 25% in comparison to PHA alone. For CrCl₃ and PdCl₂, less than 25% toxicity was observed with the highest concentration evaluated; ^cthe optimal concentration of each metal salt for induction of IL-2, IL-4 and IL-13 production was assessed by stimulation with peripheral blood mononuclear cells (PBMC) from subjects with patch test positive reactions to the metal examined. PBMC from non-allergic subjects were assessed in parallel.

(Table 2) using ELISpot and ELISA for IL-2 and IL-13, whereas IL-4 was assessed by ELISpot and IFN-γ by ELISA. The choice of assays was based on previous studies comparing the assay which was most suitable to use [12].

All metals elicited cytokine production in individual patients with patch test reactivity to the corresponding metal.

As exemplified by selected patients with significant *in vitro* responses, Ni, Co, Cr, Pd and Au had the capacity to elicit both Th1- and Th2-type cytokines (Fig. 1), whereas control PBMC displayed low production of cytokine in response to all metals (Fig. 1). A slight elevation of IL-2 production was also noted, however, in metal-stimulated PBMC from controls, in

Fig. 1. Nickel (Ni), cobalt (Co), chromium (Cr), palladium (Pd), and gold (Au) induce a similar, mixed Th1-/Th2-type cytokine response in peripheral blood mononuclear cells (PBMC) from metal allergic subjects. PBMC from patch test positive or control subjects were incubated in the absence or presence of the metal to which the subject is reactive in the patch test. Interleukin (IL)-2, IL-13 and interferon (IFN)-γ production and the number of IL-2-, IL-4- and IL-13-producing cells were determined by enzyme-linked immunosorbent assay (ELISA) (*y*-axis on the left) and enzyme-linked immunospot assay (ELISpot) (*y*-axis on the right), respectively. Plots show spontaneous cytokine production in the absence of metal stimuli and production induced by metals. Shown are responses to Ni (donor 1), Co (donor 5), Cr (donor 15), Pd (donor 13) and Au (donor 26), see Table 1. The metal to which each subject reacted in the patch test, and which was used for *in vitro* stimulation, is indicated. For comparison, the responses (mean and standard deviation) of the non-allergic control subjects (*n* = 5) are shown on the right panel of each plot.



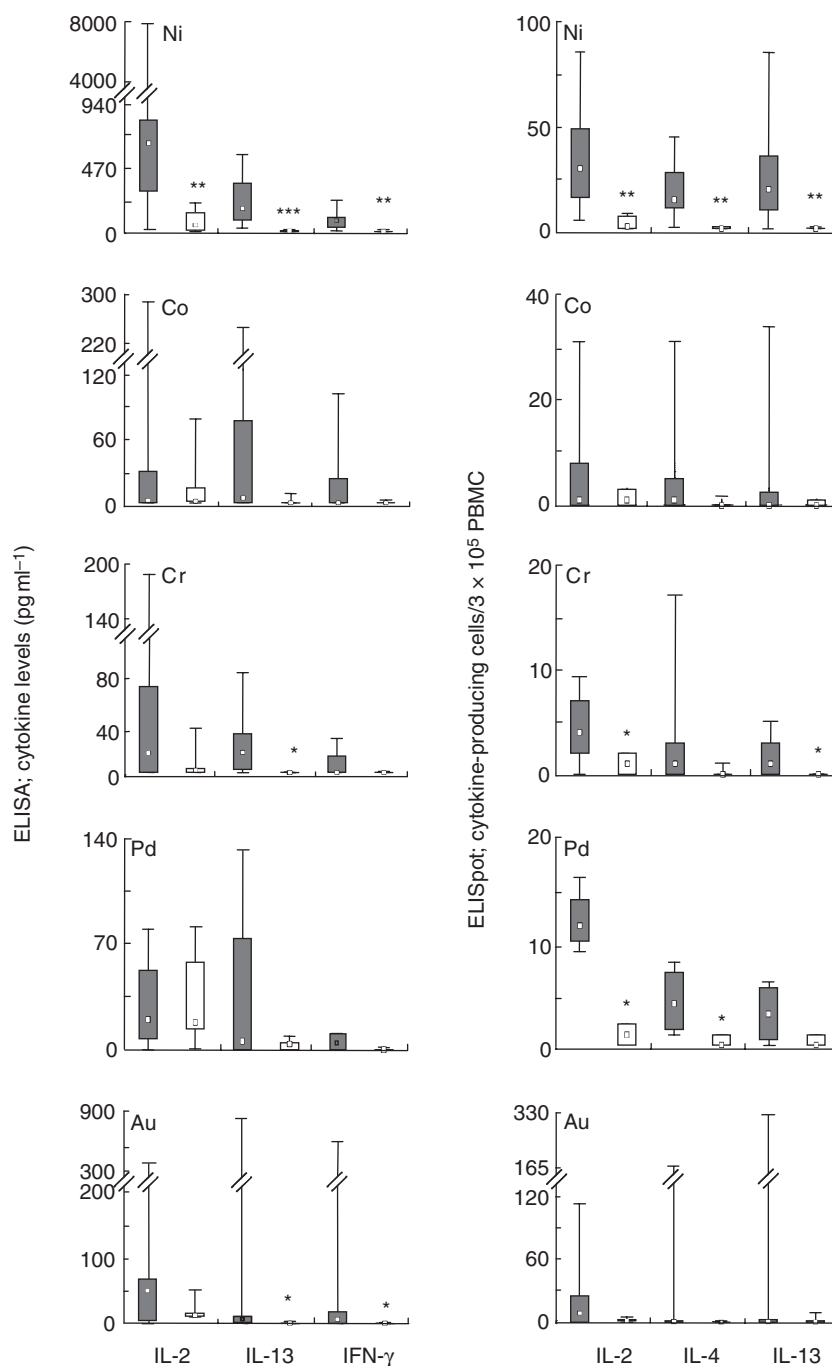


Fig. 2. Profile of cytokine responses to nickel (Ni), cobalt (Co), chromium (Cr), palladium (Pd) and gold (Au) in metal patch test positive and control subjects. Peripheral blood mononuclear cells (PBMC) from metal patch test positive (Ni, $n = 17$; Co, $n = 12$; Cr, $n = 9$; Pd, $n = 4$; Au, $n = 10$) or control ($n = 5$) subjects were incubated in the absence or presence of the metal(s) to which each metal allergic subject is reactive in the patch test. Interleukin (IL)-2, IL-13 and interferon (IFN)- γ production and the number of IL-2-, IL-4- and IL-13-producing cells were measured by enzyme-linked immunosorbent assay (ELISA) (left panel) and enzyme-linked immunospot assay (ELISpot) (right panel), respectively. Shown are data after subtraction of spontaneous background with the median response for each group indicated within the boxes (grey boxes for metal patch test positive subjects; open boxes for patch test negative non-allergic controls). The top and bottom ends of each box represent the 75th and 25th percentiles, respectively, while the top and bottom bars indicate the maximum and minimum values in the data set. The statistical differences between the groups following a Mann-Whitney U -test are depicted with asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

particular for Ni and Pd (Figs 1 and 2). When applying cut-off definition for positive *versus* negative response all patients with strong patch test reactivity to any of the metals responded to the corresponding metal, whereas subjects with moderate or weak patch test reactivity to Co, Cr, Pd and/or Au were either *in vitro* responsive or not (Table 3). Patients with a strong patch test also, in general, responded with a broader cytokine panel (this could not be defined for Pd due to the lack of patients with strong patch test reactivity) (Table 3). For Ni, all patients with a positive patch test responded *in vitro* and in most cases with multiple cytokines (Table 3). *In vitro*

reactivity to multiple metals was frequent, with concomitant reactivity to Ni and Pd being most common.

In line with the strong individual responses to Ni, we found a significant difference in the *in vitro* response, for all cytokines, between the Ni patch test positive group and the non-allergic controls (Fig. 2). Also the patient groups with reactivity to Cr, Pd and Au displayed higher responses than the controls, despite the fact that not all individuals in the groups responded *in vitro* (Fig. 2). In comparison with the controls, Cr-reactive patients responded significantly with increased IL-2 (ELISpot) and IL-13 (ELISA and ELISpot), Pd

Table 3. Th1- and Th2-type cytokine responses to metals displayed by each subject.

Subject	Ni (II) ^b	Co (II)	Cr (III)	Pd (II)	Au (I)
ACD ^a (Standard series; <i>n</i> = 16)					
1	●●●● ^c	●●●●	○○○nd	○○●●	○○○○
2	○○●●	○○○○	○○●○	○○○○	●●●●
4	○○○○	○○○○	○○●○	○○○○	●●●○
6	●●●●	●●●○	○○○○	●●●○	○○○○
7	●●●●	○○○○	○○○○	○○●○	○○○○
8	○○●●	○○○○	○○○○	○○○○	○○○○
9	●●●●	○○○○	○○○○	○○●○	○○○○
11	●○●○	○○○○	○○○○	○○○○	○○○○
15	○○●●	●○●○	●●●○	○○○○	○○○○
20	○○●○	○○●○	●●○○	○○○○	○○○○
21	○○○○	○○○○	○○○○	○○○○	○○○○
22	○○○○	○○○○	○○●○	○○○○	○○○○
24	○○○○	○○●○	○○●○	○○○○	○○○○
27	●●●●	○○○○	○○●○	○○●○	○○○○
28	○○●○	○○○○	○○○○	○○●○	○○○○
29	●●●●	○○○○	○○○○	●●●○	○○○○
ACD (Dental series; <i>n</i> = 15)					
3	○○○○	○○○○	○○○○	○○○○	●○○○
5	●●●●	●●●○	○○○○	○○●○	○○○○
10	○○○○	○○○○	○○○○	○○○○	○○○○
12	○○○○	○○○○	○○○○	○○○○	●○○○
13	●●●●	○○○○	○○○○	○○●○	●○○○
14	○○○○	○○○○	○○○○	○○○○	●○○○
16	●●●●	●○○○	○○○○	●○○○	○○○○
17	○○○○	○○○○	○○○○	○○○○	○○○○
18	●●●●	○○●○	○○○○	○○○○	○○○○
19	●○○○	○○○○	○○○○	○○●○	○○○○
23	○○●○	○○○○	○○○○	○○○○	○○○○
25	○○●○	○○○○	○○○○	○○○○	○○○○
26	○○●○	○○○○	○○○○	○○○○	●●●●
30	●●●●	○○○○	○○○○	○○○○	○○○○
31	○○●○	○○○○	○○○○	○○●○	○○○○
Controls					
1-5 ^d	○○○○	○○○○	○○○○	○○○○	○○○○

^aSubjects with *in vivo* patch test reactivity to one or several metals included in the standard or dental test series. *In vitro* responses to metals were defined by comparison to cut-off definitions based on the increment and ratio computed on the spontaneous (background) cytokine production in the unstimulated cells (see Materials and methods). ^bNi (II) refers to NiCl₂. ^cThe four symbols from left to right represent interleukin (IL)-2 [enzyme-linked immunospot (ELISpot) and/or enzyme-linked immunosorbent assay (ELISA)], IL-4 (ELISpot), IL-13 (ELISpot and/or ELISA) and interferon (IFN)-γ (ELISA), respectively, for each subject and metal. ● = response above cut-off for both cytokine increment and ratio; ○ = below cut-off for cytokine increment and/or ratio. Only subjects responding above cut-off for both cytokine increment and ratio (●) were considered positive in the *in vitro* cytokine assays. ^dAll the control subjects were negative according to the cut-off definition. For responses to Cr in donor 1, n.d. = not done by ELISA; all samples were, however, tested with the complete panel of metals in ELISpot. Shaded boxes indicate that the patient had positive patch test reactivity with the metal in question.

patch test positive patients by IL-2 (ELISpot) and IL-4 (ELISpot) and Au reactive patients by IFN-γ (ELISA) and IL-13 (ELISA). Although individual patients in the Co group responded to Co with both Th1- and Th2-type cytokines (Table 3), and patient responses at a group level tended to be higher than the controls, no significant differences between the groups were found (Fig. 2).

Among 52 metal reactions identified by patch test in the patients, *in vitro* reactivity to the corresponding metals were found in 37 cases including 21 of 21 of the strong, 12 of 21 of the moderate and four of 10 of the weak patch test reactions; 15 patch test reactions were not matched by an *in vitro* response (Table 3). The highest proportion of patch test responses that were not matched *in vitro* were in the Co group where six of 12 patients did not respond, although 10 of them displayed *in vitro* reactivity to Ni.

A number of the metal patch test positive patients, but none of the controls, also reacted *in vitro* with metals to which they had a negative patch test. This was most common in the Ni patch test negative patient group, where five patients reacted *in vitro* with Ni. *In vitro* reactivity with other metals in the absence of a matching patch test reactivity were found in two cases for Co, one for Cr, two for Pd and none for Au. It is noteworthy that, of these 10 *in vitro* positive, but patch test negative cases, eight displayed significant reactivity manifested by induction of multiple cytokines.

The time interval between the patch test and the *in vitro* test in the subjects ranged from 1 week to 48 months, but there was no indication that subjects with a recent patch displayed higher *in vitro* responses, or that subjects with a longer time interval between the tests responded less well *in vitro*.

Discussion

In this study we demonstrate that Co, Cr, Pd and Au, similar to Ni, elicit both Th1- and Th2-type cytokines *in vitro* in PBMC from patients with patch test reactivity to the respective metals. The previous lack of general information on the cytokine response profile to metals involved in ACD, other than Ni, may be explained partly by a greater research interest in Ni due to the high prevalence of ACD to Ni. However, some studies have assessed the capacity of other metals to induce selected cytokines in PBMC from allergic subjects. For example, IL-5 and IFN-γ responses to Co and Cr [34] and IFN-γ responses to Cr [35] have been demonstrated in PBMC from allergic subjects. In those studies, co-stimulatory cytokines or mitogen, respectively, were added together with the metals in order to induce detectable levels of cytokines.

In the present study, metal-induced cytokine profiles were assessed by *in vitro* stimulation of PBMC by metal salts alone. Individual patients with a positive patch test and a high responsiveness *in vitro* reacted with the corresponding metal by IL-2 and/or IFN-γ as well as with IL-4 and/or IL-13 production, demonstrating the involvement of both Th1-

and Th2-type cytokines in the response to all these metals. At a group level, comparing patch test positive patients and control subjects, all metals except Co elicited significantly higher production of both Th1- and Th2- type cytokines in PBMC from the patients. That not all cytokine responses differed significantly at a group level was not surprising considering that, with the exception of the Ni group, several metal patch test positive patients failed to react *in vitro* with the corresponding metal.

The finding that not only Ni, but also other metals, elicits a mixed Th1- and Th2-type of response in PBMC from sensitized subjects provides further evidence that the immunological response to contact allergens differs from the initial description of ACD in humans as a condition involving primarily Th1-type responses. A similar mixed Th1- and Th2-type response induced by methylchloroisothiazolinone/methylisothiazolinone in sensitized patients suggests that this type of immunological response is not limited to metal haptens but also extends to organic molecule haptens [36].

In vitro reactivity with multiple metals was frequently observed in this study, in line with many previous observations based on patch testing [4,16–19,22]. Except for some patients who reacted exclusively with Ni, Au and in one case Cr, other patients displayed reactivity with multiple metals. Concomitant *in vitro* reactivity to Ni and Pd was most common; all 12 patients who reacted *in vitro* to Pd also reacted to Ni. With regard to patch test reactivity to Pd, it has been reported to be found almost exclusively in subjects with reactivity to Ni [14,16]. Considering the reported cross-reactivity between Ni and Pd [21], and the fact that *in vitro* responses to Ni were stronger in general, it is thus possible that the Pd reactivity observed is caused predominantly by Ni-induced T cells. Concomitant reactivity to Ni and Co has, instead, been suggested to be due to co-induction [21]. We found that seven of eight patients reactive with Co *in vitro* also reacted with Ni. However, we also found that five of the six Co patch test reactive patients who did not react with Co *in vitro* displayed *in vitro* reactivity to Ni. This is notable, as some studies based on patch testing have suggested that isolated Co reactivity is rare [37], with Co reactivity in some subjects attributed to Ni contamination of the Co patch test material [17,38].

The *in vitro* response to metals agreed well with the patch test data for the patients with strong patch test reactivity, i.e. all those patients reacted *in vitro* with the corresponding metal. With regard to Ni, *in vitro* responses were also observed in patients with lesser patch test reactivity. The responses of patients with moderate and weak patch test reactivity to Co, Cr, Pd or Au were not always consistent with the patch test data, i.e. they could either be positive or negative regardless of the patch test reactivity. However, given the well-documented problems associated with patch testing with these metals and other compounds [17,38,39], caution is needed when comparing *in vitro* responses and patch test data in subjects with weaker patch test reactivity. Cr, Au and

Co, together with many other substances used commonly for patch testing, are also known to have an inherent irritative capacity and often elicit irritative and/or doubtful reactions difficult to distinguish from weak but true positive reactions [39,40]. It is thus not unlikely that subjects with weaker patch test reactivity fail to react *in vitro* due to a lack of true immunological reactivity. However, the possibility that a true ACD reaction in these subjects is not identified by *in vitro* assays has also to be considered. The fact that responses to Ni are detected more readily by *in vitro* assays may suggest that the immunological reactivity to other metals is, in general, of lower magnitude.

Whereas the non-allergic patch test negative controls did not react significantly to any metal *in vitro*, in some cases subjects with ACD and patch reactivity to one or several metals displayed *in vitro* responses to metals to which they tested negative in the patch test. This is probably not due to non-specific induction of cytokines, as non-allergic controls did not respond to any metal *in vitro*. However, it is possible that PBMC from subjects with an immunological reactivity to any given metal are triggered more easily to react with other metals in a non-specific manner. On the other hand, the *in vitro* reactivity may reflect a true immunological reaction that was not identified by the patch test. This is indicated by the fact that eight of 10 *in vitro* positive reactions to metals in patients with a negative patch test to the corresponding metal included significant production of multiple cytokines. It is well documented that the reproducibility of weak patch test results is poor and reports of false positive as well as false negative results are frequent, whereas similar studies based on *in vitro* methods have not been conducted so far [39,41]. To elucidate the discrepancy between weaker patch test data and *in vitro* cytokine responses of lower magnitude, follow-up studies are required. Such studies would also be of high relevance for the possible development of future *in vitro*-based diagnostic assays for ACD.

In conclusion, our results suggest that sensitization to Co, Cr, Pd and Au involves an immune response similar to the mixed Th1- and Th2-type cytokine profile shown previously to be induced by Ni.

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