

ORIGINAL PAPER

Detection of chromium allergy by cellular *in vitro* methodsM. Lindemann*, F. Rietschel[†], M. Zabel[†] and H. Grosse-Wilde**Institut für Immunologie, Universitätsklinikum Essen and [†]Hautklinik, Knappschafts Krankenhaus Recklinghausen, GermanyClinical and
Experimental
Allergy

Summary

Background The standard assay for the detection of chromium sensitization, the patch test, does not allow discrimination between patients with and without clinical symptoms of allergy.

Objective The aim of this study was to prove whether cellular *in vitro* tests are predictive of chromium allergy.

Methods Chromium-sensitized volunteers with and without clinically manifest allergy and non-sensitized healthy controls ($n = 37$, 19, and 26, respectively) were analysed by cellular *in vitro* methods using tri- and hexavalent chromium (chromium chloride and potassium dichromate) as stimuli. The results were correlated with clinical and anamnestic data.

Results Sensitized individuals with an allergy displayed significantly higher lymphocyte transformation test (LTT) responses than sensitized volunteers without allergy and controls ($P < 0.05$ and $P < 0.01$, respectively). 12.5 µg/mL of chromium chloride and 50 ng/mL of potassium dichromate were found to be optimal to discriminate between sensitized individuals with and without allergy. Combining the results of chromium chloride and potassium dichromate LTT, a positive reaction to at least one of the stimuli was highly predictive of allergy [sensitization with vs. without allergy: Odds ratio (OR) = 6.4, $P = 0.004$; sensitization with allergy vs. controls: OR = 11.5, $P < 0.0001$]. On the contrary, IFN- γ , IL-2, IL-4, IL-10, and IL-12 production to the ELISpot, patch test results, sensitization against other metals, and atopy score did not significantly discriminate between sensitization with and without allergy. However, IFN- γ responses towards chromium chloride were significantly correlated with the strength of patch test reactivity ($r = 0.49$, $P = 0.002$). By IFN- γ ELISpot, the average precursor cell frequency reactive to trivalent chromium could be defined as 26, 15, and 11 : 10⁶ in volunteers with sensitization and allergy, with sensitization without allergy, and controls, respectively.

Conclusions In contrast to the patch test, the LTT appears to be a method that is predictive of chromium allergy.

Keywords chromium allergy, ELISpot, *in vitro* cytokine production, *in vitro* lymphocyte proliferation, patch test

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Introduction

Chromium is the fourth frequent element worldwide and is the third frequent cause for metal sensitization [1]. In the general population, the frequency of chromium sensitization is estimated as 0.6–1.5% [2]. Sensitization correlates with exposure to trivalent or hexavalent chromium, which is used by workers in the tanning, pottery, or metal industry, and by construction workers [3, 4]. In the case of e.g. construction workers, sensitization is caused by the water-soluble hexavalent chromate, which is one compo-

nent of cement, a substance with alkaline, abrasive and irritant properties [5]. Professional exposure leads to an increase in the sensitization rate to 20% [6]. However, in some Scandinavian countries sensitization rates have currently been decreasing due to the addition of ferrous sulphate to cement, which was started more than 20 years ago [7]. Since 2005, there has been a similar regularization in the European Union [8]. Patch testing is the gold standard for the diagnosis of chromium sensitization. To diagnose chromium allergy, however, specific skin symptoms and environmental exposure are also taken into

account. Irritant reactions to the patch test can frequently hamper the diagnosis of chromium sensitization, e.g. when using the standard concentration of 0.5% chromium dichromate, as many as one out of two positive reactions are considered to be an irritant [1]. Furthermore, in chronic cases allergic and irritant contact dermatitis can hardly be differentiated by clinical symptoms [9]. Thus, sensitization against chromium cannot be equated with clinically manifest allergy and a positive patch test result is of limited clinical significance [10].

It is commonly assumed that trivalent chromium forms the actual hapten, whereas chromate, containing hexavalent chromium, functions as a pro-hapten [11]. Chromate is most capable of penetrating the epidermal layers, whereas trivalent chromium strongly binds to proteins [12]. As tri- and hexavalent chromium ions seem to be involved in sensitization, we decided to use both chromium compounds as stimuli for the *in vitro* assays.

In contrast to previous studies that compared either allergic [13–15] or patch test-positive volunteers [16] with healthy controls, we considered three different groups: with sensitization with allergy, with sensitization without allergy, and non-sensitized healthy controls. We wanted to know whether cellular *in vitro* responses could discriminate between sensitized individuals with and without clinically manifest allergy.

Here, we present our data on 56 chromium-sensitized volunteers, that show for the first time that the strength of LTT reactions towards chromium chloride and potassium dichromate was predictive of allergy.

Materials and methods

Study subjects

In total, 82 volunteers from the Department of Dermatology, Knappschaftskrankenhaus Recklinghausen, answered questionnaires concerning exposure to metals, especially chromium, and clinical signs of allergy. They were all examined by patch and cellular *in vitro* tests. By a patch test, 26 were defined as non-sensitized (healthy controls) and 56 as sensitized. None of these healthy controls was sensitized against any metal or suffered from skin disease. In the group of sensitized volunteers, 37 reported an anamnesis and symptoms typical of an allergy against chromium (allergic contact dermatitis) [17], whereas 19 did not (Table 1). Following patch testing, 40 mL heparinized blood and 5 mL serum were collected for cellular *in vitro* tests and the measurement of total IgE. The interval between patch testing and venipuncture took 7 days to 17 years (median 63 months). The study was approved by the local ethical committee and it had been conducted according to the principles expressed in the Declaration of Helsinki; all volunteers provided written informed consent to participate.

Questionnaire on exposure to metals and clinical signs of allergy

The questionnaire recorded the age, sex, time-point of a previous patch test, professional or hobby exposure to metals, skin diseases, hyperhidrosis, and clinical signs of allergy. With the help of a medical examination and a face-to-face interview by an experienced dermatologist symptoms of allergies that occurred long time ago were also detected.

Patch testing

Potassium dichromate (0.5% in vaseline) was applied by Haye's test chambers (Haye's Service B. V., Alphen aan den Rijn, the Netherlands) to the healthy, non-inflamed back of volunteers [17]. After 24 or 48 and 72–144 h reactions were classified according to the standard criteria of the International Contact Dermatitis Research Group [18]. Patch test results after 24 and 48 h occlusion differed as expected; a positive reaction was observed in 25% and 70% of the volunteers, respectively, at the first reading. However, the ratio of tests read after 24 and 48 h did not differ significantly between sensitized volunteers with and without clinical manifestation/symptoms of allergy. A crescendo character in patch test results was considered to be typical for allergic reactions, and a decrescendo character for toxic-irritable reactions. The maximum strength of reactions was classified as weak (1+), moderate (2+), and strong (3+). The maximum strength of patch test reactions was taken for correlation analyses.

Atopy score

Atopic skin diathesis was recorded based on 24 anamnestic and clinical criteria according to Diepgen et al. [19], e.g., Hertoghe's sign, palmar hyperlinearity, white dermographism, seborrhea, and total IgE. Data were scored and added. Score values of ≥ 10 were considered to be positive.

Chromium-specific lymphocyte transformation test

Peripheral blood mononuclear cells (PBMCs) from heparinized blood were separated by Ficoll-HypaqueTM (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. 2×10^5 PBMC in 200 μ L culture volume were stimulated with tri- or hexavalent chromium at concentrations of 6, 9, 13, 19, 25, 38, and 50 μ g/mL (chromium (III) chloride hexahydrate, 99.995%, Sigma-Aldrich, Milwaukee, WI, USA) or 25, 38, 50, 75, 100, 150, and 200 ng/mL (potassium dichromate >99.5%, crystalline, Roth, Karlsruhe, Germany), respectively, in round-bottom microtitre plates (Becton Dickinson Labware, Heidelberg, Germany). Cell cultures were set as

Table 1. Baseline characteristics of chromium-sensitized volunteers

	With allergy (<i>n</i> = 37)	Without allergy (<i>n</i> = 19)	<i>P</i>
Median age (range), years	52 (36–75)	49 (25–79)	0.6*
Sex (female/male), no.	21/16	9/10	0.6 [†]
Interval: patch test – venipuncture, no. (%)			0.3 [‡]
≤2 month	4 (11)	4 (21)	
>2 month and ≤1 year	7 (19)	2 (11)	
>1 year and ≤4 years	8 (22)	1 (5)	
>4 years	18 (48)	12 (63)	
Median interval (range), months	62 (0.2–186)	64 (0.2–202)	0.7*
Plateau/crescendo reaction to patch test, no. (%)	27 (64)	11 (58)	0.4 [†]
Strength of patch test reactivity, no. (%) [§]			0.2 [‡]
1+	9 (24)	2 (10)	
2+	20 (54)	12 (63)	
3+	5 (14)	0 (0)	
Professional exposure to chromium, no. (%)	22 (59)	4 (21)	0.01 [†]
Hobby exposure to chromium, no. (%)	8 (22)	5 (26)	0.7 [†]
Sensitization against metals other than chromium, no. (%)			
Cobalt	10 (27)	4 (21)	0.8 [†]
Nickel	11 (30)	9 (47)	0.2 [†]
Any	17 (46)	11 (58)	0.6 [†]
Sensitization against metals, mean no. ± SEM	1.6 ± 0.3	2.4 ± 0.6	0.4*
Skin disease, no. (%)	17 (46)	8 (42)	1.0 [†]
Hyperhidrosis, no. (%)	11 (30)	6 (32)	1.0 [†]
Total IgE (kU/L), mean ± SEM	169 ± 45	198 ± 102	1.0*
Score of atopy, mean ± SEM	10.8 ± 0.9	9.3 ± 0.9	0.2*

Data were analysed by

*Mann–Whitney test, [†]Fisher's exact test, or [‡]χ²-test.

[§]In three sensitized volunteers with allergy and five without allergy, data on the strength of reactivity were not available. In most of these cases, patch testing was performed >10 years ago and the detailed protocol was not archived.

sextriplicates. The culture conditions and measurement of H3-TdR uptake followed a protocol described previously [20]. To optimize the LTT, kinetic experiments were performed for 6, 7, and 8 days. LTT results were expressed as counts per minute (CPM) increment, i.e. lymphocyte transformation with stimulation minus autologous control (stimulation only with culture medium). In addition, stimulation indices (SI) were considered (quotient of stimulated and autologous proliferation).

Chromium-specific ELISpot assay

Four × 10⁵ PBMC in 200 µL culture volume were pre-incubated with tri- or hexavalent chromium in round-bottom microtitre plates at concentrations that were also used for the LTT experiments. After 48 h the cells were transferred to MultiScreen-HA ELISpot plates (MA-HAN4550, Millipore, Bedford, MA, USA). Subsequent cytokine production (IFN-γ, IL-2, IL-4) was detected using a membrane-anchored first antibody and a biotinylated second antibody as published [20]. The production of

IL-10 and IL-12 was measured using antibodies at the following concentrations: purified monoclonal anti-human IL-10 (clone 9D7, 10 µg/mL) and biotinylated anti-human IL-10 (clone 12G8, 1 µg/mL), purified monoclonal anti-human IL-12 (clone IL12-I, 10 µg/mL), and biotinylated anti-human IL-12 (clone IL12-II, 2 µg/mL) (Mabtech, Nacka, Sweden). After a further 48 h incubation in ELISpot plates, the resultant spots were quantified using an ELISpot plate reader (Bioreader 2000, Biosys, Karben, Germany). Results were presented as incremental spots.

Statistical analysis

Continuous variables were expressed as the mean and standard error of the mean (SEM) if not otherwise stated. The Mann–Whitney *U* test, Fisher's exact test, or χ²-test was used to compare different groups as appropriate. We calculated the sensitivity and specificity from the 2-by-2 contingency tables. The Spearman rank sum test was used for correlating variables. GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA, USA) was

applied for all calculations. P -values of <0.05 were considered to be statistically significant.

Results

Chromium chloride and potassium dichromate induced a dose-dependent lymphocyte transformation response in volunteers with sensitization with allergy, with sensitiza-

tion without allergy, and in non-sensitized healthy controls (Fig. 1). Here, 7- and 8-day LTT cultures showed greater differences between these three groups than 6-day cultures. Of note, in 7- and 8-day cultures, sensitized volunteers with vs. without allergy displayed significantly ($P < 0.05$) higher proliferative responses towards both chromium compounds. Furthermore, the responses in allergic volunteers were significantly higher ($P < 0.01$)

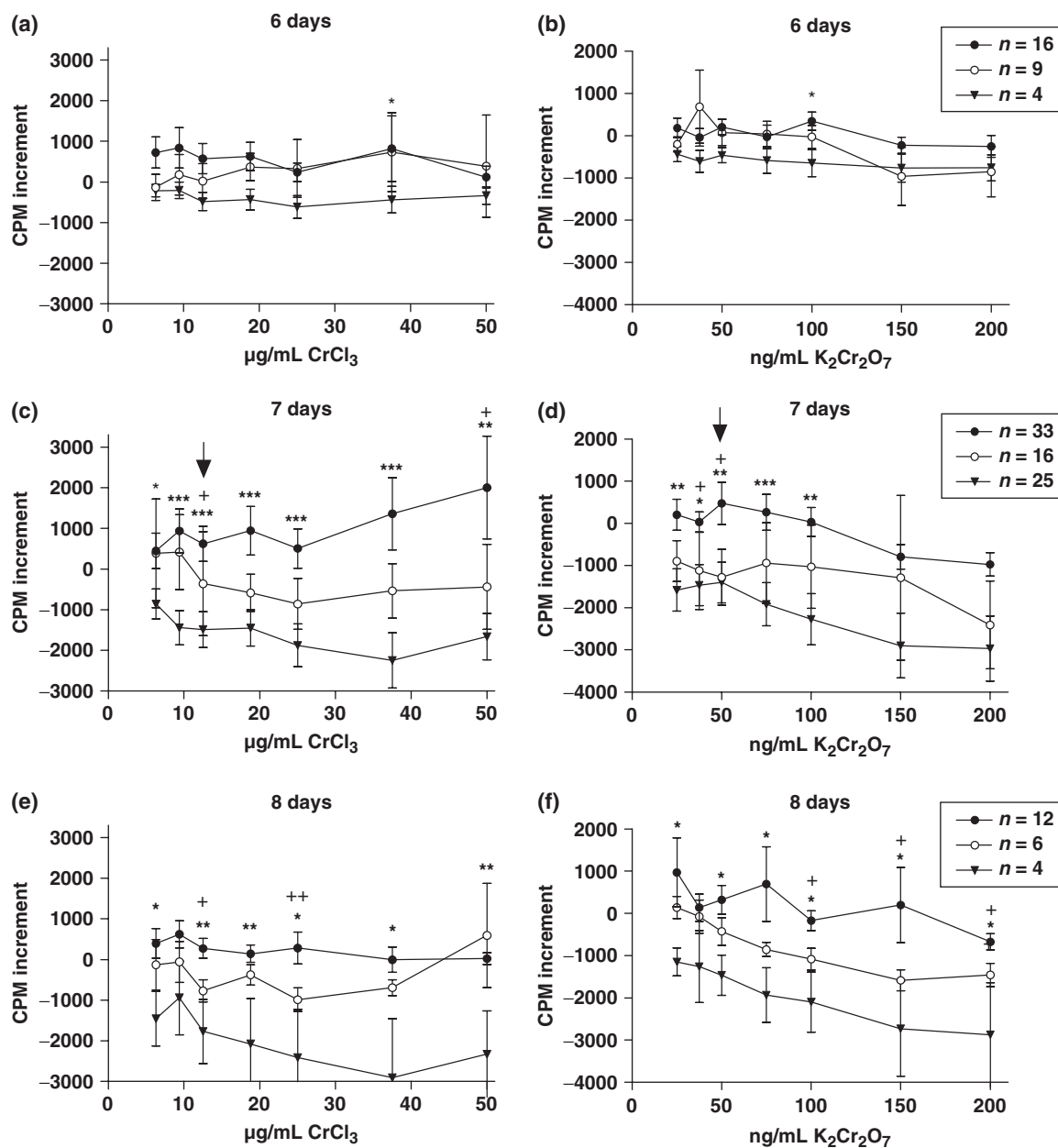


Fig. 1. Dose-response curves of lymphocyte transformation test (LTT) results using seven different concentrations of chromium chloride (CrCl_3) and potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) in 6-day (a, b), 7-day (c, d), and 8-day (e, f) cell cultures. Data from 37 sensitized volunteers with allergy (filled circles), 19 without allergy (open circles), and 26 non-sensitized healthy controls (triangles) are given as mean $\pm$ standard error of the mean (SEM). 4/37, 3/19, and 1/26 volunteers in the respective groups were only tested in a 6-day or an 8-day but not in a 7-day cell culture. Arrows indicate culture conditions found to be optimal to discriminate between sensitized individuals with and without chromium allergy. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for sensitized volunteers with allergy vs. controls or + $P < 0.05$ and ++ $P < 0.01$ for sensitized volunteers with vs. without allergy (Mann-Whitney test).

than that in healthy controls. Maximum proliferative responses were higher in 7- than in 8-day cell cultures. 12.5 µg/mL of chromium chloride and 50 ng/mL of potassium dichromate were found to be optimal to discriminate between sensitized individuals with and without allergy in 7-day cell cultures (Figs 1c and d). Whereas lymphocyte proliferation was stimulated in allergic volunteers, no proliferation above background (autologous) values could be detected in sensitized volunteers without allergy as well as in controls. In the latter two groups, the mean CPM increment values were negative, i.e. tri- and hexavalent chromium appeared to be toxic to the cell cultures.

Using 12.5 µg/mL of chromium chloride (or 50 ng/mL of potassium dichromate, respectively, data given in parenthesis) as a stimulus in 7-day cultures, 18 (16) out of 33 volunteers with sensitization with allergy, five (3) out of 16 with sensitization without allergy, and four (3) out of 25 non-sensitized healthy controls displayed a positive reaction. Combining the results of chromium chloride and potassium dichromate LTT, a positive reaction to at least one of the stimuli was observed in 23/33 (70%), 5/16 (31%), and 5/25 (20%) volunteers, respectively. Thus, a positive reaction to either of the chromium compounds is highly predictive of allergy (sensitization with vs. without allergy: OR = 6.4, $P = 0.004$; sensitization with allergy vs. controls: OR = 11.5, $P < 0.0001$). Depending on the group used for comparisons, allergy could be predicted with a sensitivity of 70% and a specificity of 74% or 70% and 83%, respectively (comparison with sensitized volunteers without allergy or with controls). Furthermore, analysing all sensitized individuals, 28/49 (57%) were classified as positive. Notably, Spearman's analysis showed that cellular *in vitro* responses towards chromium chloride and potassium dichromate were significantly correlated ($r = 0.73$, $P < 0.0001$; 7-day culture, 12.5 µg/mL and 50 ng/mL, respectively).

Moreover, LTT responses expressed as CPM increment and SI were significantly correlated ($r = 0.85$, $P < 0.0001$ and $r = 0.86$, $P < 0.0001$; 7-day culture, 12.5 µg/mL chromium chloride and 50 ng/mL potassium dichromate, respectively). But considering the SI instead of CPM increment values as depicted in Fig. 1, differences between sensitized volunteers with vs. without allergy just escaped statistical significance (data not shown).

In contrast to the LTT, the ELISpot assay was of minor value for the prediction of an allergy against chromium. Volunteers with sensitization with and without allergy did not differ significantly in terms of IFN- γ , IL-2, IL-4, IL-10, and IL-12 production following stimulation with tri- and hexavalent chromium compounds. Combining the results of chromium chloride and potassium dichromate ELISpot (IFN- γ , IL-2, IL-4, IL-10, and IL-12) in the same way as described above for the LTT, a positive reaction to at least one of the stimuli was not predictive of chromium allergy. However, the mean IFN- γ , IL-4, and IL-10 production

tended to be increased in volunteers with vs. without allergy. In addition, allergic volunteers displayed significantly ($P < 0.05$) higher IFN- γ production following stimulation with chromium chloride [50 µg/mL] than healthy controls (4.5 ± 1.7 vs. 0.1 ± 0.4 spots increment) (Fig. 2). A similar trend could be observed for IL-4 and IL-10 production. Furthermore, LTT and ELISpot results were only weakly correlated. Based on maximum IFN- γ production, an average precursor cell frequency reactive to trivalent chromium can be defined as 10.4, 6.0, and 4.2 per 4×10^5 PBMC (or 26, 15, and 11 per 10^6 PBMC) in volunteers with sensitization with allergy, with sensitization without allergy, and healthy controls, respectively (Fig. 2). Interestingly, IFN- γ responses towards chromium chloride were significantly correlated with the strength of patch test reactivity ($r = 0.49$, $P = 0.002$) (Fig. 3). In

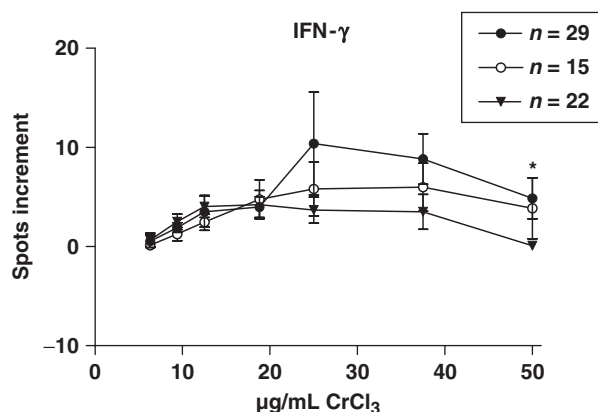


Fig. 2. Dose-response curves of IFN- γ ELISpot results using seven different concentrations of chromium chloride (CrCl₃). Data in sensitized volunteers with allergy (filled circles), without allergy (open circles), and non-sensitized healthy controls (triangles) are given as mean $\pm$ standard error of the mean (SEM). * $P < 0.05$ for sensitized volunteers with allergy vs. healthy controls (Mann-Whitney test).

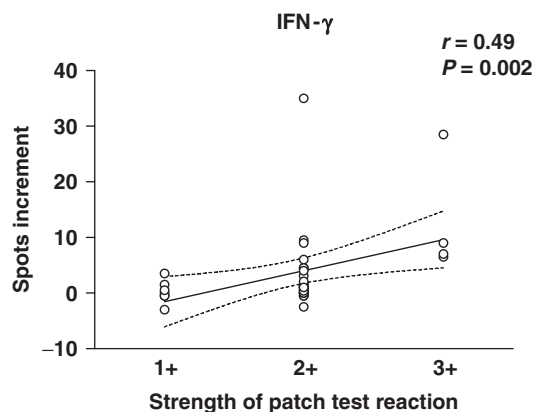


Fig. 3. Spearman correlation analysis between ELISpot results and maximum patch test reactivity in chromium-sensitized volunteers ($n = 38$). IFN- γ responses were measured after stimulation with chromium chloride (12.5 µg/mL). The continuous line represents the regression line, and the fractioned ones represent the 95% confidence interval.

contrast, patch test reactivity showed no correlation with LTT responses. Taken together, clinically manifest allergy correlates with LTT responses and patch test results with ELISpot data, but not vice versa.

Professional exposure to chromium was the only baseline characteristic that differed significantly ($P=0.01$) between sensitized volunteers with and without allergy (Table 1). All the other baseline characteristics (age, sex, interval from patch test to venipuncture, characteristics of the patch test reaction (plateau/crescendo and strength), hobby exposure to metals, co-sensitization against other metals, skin diseases, hyperhidrosis, total IgE, or score of atopy) did not differ significantly. Moreover, the interval from patch test to venipuncture had no impact on the strength of chromium-specific lymphocyte proliferation or cytokine production.

Finally, the diagnosis of an allergy against chromium appears to be very likely if three criteria are fulfilled: (1) positive patch test (verification of sensitization), (2) positive reaction to the LTT, and (3) professional exposure to chromium. In our cohort, the combined presence of the three criteria significantly ($P<0.0001$) predicts allergy with an OR of 54.7 and a specificity of 100% (41/41). Using these close criteria, the sensitivity, however, is low (39%). Thus, the combined evaluation appears to be an excellent tool to assure the diagnosis of chromium allergy.

Discussion

We focused in this report on the discrimination between chromium-sensitized individuals with and without clinical manifestation/symptoms of allergy using cellular *in vitro* methods. This study comprises considerably more sensitized individuals ($n=56$) than previous studies and it is, therefore, suitable to define predictors of clinically apparent allergy. We could show that proliferative responses to chromium chloride and potassium dichromate significantly ($P<0.05$) predicted allergy, whereas production of the cytokines IFN- γ , IL-2, IL-4, IL-10, and IL-12 did not. Clinical characteristics such as plateau/crescendo course and the strength of reactivity to the patch test or co-sensibilization against other metals, however, could not predict allergy in our cohort.

Both trivalent and hexavalent chromium induced proliferation and cytokine production to a similar degree. However, for comparable results, an approximately 250-fold higher concentration of chromium chloride is needed, compared with potassium dichromate. This weaker T-cell reactivity to chromium chloride can be explained by the earlier findings of Al-Tawil *et al.* [16] and Räsänen *et al.* [13], who demonstrated that trivalent chromium binds strongly to proteins, most of which converts to a non-immunogenic form [21]. Apart from the induction of specific lymphocyte proliferation, chromium compounds appear to be toxic, a phenomenon that we observed

especially in sensitized volunteers without allergy and healthy controls (Fig. 2). The cytotoxicity of tri- and hexavalent chromium on *in vitro* cell cultures had been described previously [22], and the underlying mechanism was reviewed recently [23]. A series of studies indicates that chromium compounds induce oxidative stress through enhanced production of reactive oxygen species, leading to genomic DNA damage and oxidative deterioration of lipids and proteins [23].

Several previous studies used the LTT method to discriminate either between chromium-allergic or sensitized volunteers and healthy controls [13–16]. However, most of the studies were small ($n=8, 8, 5$, and 31 allergic/sensitized individuals, respectively) and none of them addressed the question as to whether sensitized patients with and without clinically manifest allergy were different in terms of their cellular *in vitro* reactions. Our cell culture conditions were similar to previous publications, that also described the best results for 7- and 8-day incubations [13, 16], and similar concentrations of chromium chloride [13, 16] and potassium dichromate [13, 14, 16]. Moreover, Al-Tawil *et al.* [16] observed positive LTT reactions in 20/31 (65%) of chromium-sensitized volunteers, which is a proportion similar to our own data (57%). Extending this previous observation, we were able to show that the strength of proliferative responses depended significantly on the clinical manifestation of chromium allergy.

It had been recommended previously [24] that a minimum interval of two months between the patch test and LTT should be adhered to. This recommendation was followed in the majority of volunteers. In sensitized volunteers, the median interval from patch testing to venipuncture was rather long (63 months). Only 4/37 of sensitized volunteers with allergy and 4/19 without allergy were tested within the first two months by *in vitro* methods. Besides, responses within this interval were comparable to responses measured thereafter.

Recently, Minang *et al.* [25] compared the cytokine profile in nine chromium-sensitized volunteers and five healthy controls. By ELISpot, they observed a significantly increased IL-2 and IL-13 and a non-significantly increased IL-4 production in sensitized volunteers. Their ELISA results confirmed the ELISpot data. When going into details, only 2/9 displayed a positive IL-2, 5/9 a positive IL-13, and 2/9 a positive IL-4 response towards chromium chloride. Positive ELISpot or ELISA results could only be observed in volunteers with strong (3+) or moderate (2+), but never with weak (1+) positive patch test reactions. Furthermore, as measured by ELISA, they reported slightly increased chromium-specific IFN- γ levels in sensitized volunteers. In contrast, Moed *et al.* [11] observed no differences in chromium-specific cytokine levels (IFN- γ and IL-5 ELISA) in seven chromium-sensitized patients vs. seven controls. Our own ELISpot data in a larger cohort have indicated that IL-2 production

does not differ between sensitized volunteers and healthy controls whereas IFN- γ and IL-4 production was slightly increased in the first group. Of note, IFN- γ but not IL-2, IL-4, IL-10, or IL-12 production was significantly correlated with the strength of patch test reactivity. These contrasting findings are most likely due to the small cohorts, various experimental settings (incubation period, ELISA vs. ELISpot), and a moderate effect. When using modified cell culture conditions for the ELISA or ELISpot [11, 26], it could be observed that IFN- γ and IL-4 production were increased in sensitized/allergic patients vs. controls, respectively. In these experiments, PBMC were either stimulated by chromium compounds plus cytokine cocktails or plus phytohaemagglutinin. Thus, our observation that chromium-specific IFN- γ and IL-4 production was slightly increased in sensitized/allergic volunteers is at least similar to previous results [11, 25, 26]. With regard to the analysis of IL-12 production, the absence of any correlation with sensitization/allergy was as expected. It appears as unlikely that this cytokine would be produced by hapten-reactive T-cells.

In conclusion, according to our results, we can state that the LTT appears to be superior to the ELISpot in discriminating between chromium-sensitized individuals with and without allergy. However, it has to be confined that our ELISpot conditions may not be optimal. In particular, an increase in the cell incubation time as published by Keating et al. [27] could have led to the identification of additional chromium-reactive T-cell phenotypes (i.e. central memory T-cells). In combination, patch test, LTT, and an anamnesis of professional exposure can predict chromium allergy with a specificity of 100%. Thus, the LTT could be defined as an excellent method to confirm the diagnosis of chromium allergy, e.g. when an expert opinion is requested due to occupational dermatosis.

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