

Lymphocyte proliferation testing in chromium allergic contact dermatitis

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Summary

Background. Lymphocyte proliferation testing (LPT) has some advantages over patch testing to diagnose allergic contact dermatitis. It is harmless, objective and can be used in clinical situations where patch testing is not recommended. Unfortunately, significant success has only been achieved with nickel. There are few studies on chromium LPT and they were performed with different methods, leading to inconsistent results.

Methods. To determine the best parameters for chromium LPT, we tested 20 patients with allergic contact dermatitis to the metal and 20 controls, using various protocols.

Results. The best sensitivity and specificity ratios were achieved with 6-day cultures stimulated with a range from 7.5×10^{-4} to 5×10^{-3} mol/L of nonfiltered chromium chloride solutions. The sensitivity, specificity and accuracy values found within this range were 65%, 95% and 80%, respectively.

Conclusion. Further investigation is necessary to achieve better sensitivity values.

Introduction

Allergic contact dermatitis (ACD), a T cell-mediated cutaneous inflammatory response to low-molecular-weight chemicals, is a common and often disabling disorder. Patch testing remains the gold standard test to confirm ACD and to identify the specific causative allergen. However, it is time-consuming, subjective and not completely safe.^{1–5} The frequency and morbidity of ACD and the problems related to patch testing requires the development of an alternative test.⁶ Lymphocyte proliferation testing (LPT) is an *in vitro* assay that evaluates lymphocyte antigen recall. Owing to its better safety profile, LPT may be used when patch testing is contraindicated.^{1,7} However, good results have only been achieved with nickel, an allergen rarely associated with diagnostic difficulty.^{7–9}

Chromium, an elemental metal found in various oxidation states (+3 and +6), is an important cause of contact dermatitis among construction workers¹⁰ and workers exposed to chrome-tanned leather products.¹¹ Hexavalent [Cr(VI)] salts are stronger allergens than trivalent [Cr(III)] compounds, partly due to the greater skin permeation of the former.^{11,12} However, it has been speculated that Cr(III) is the actual hapten, as Cr(VI) is reduced to Cr(III), which in turn binds intracellular proteins, forming immunogenic complexes.¹³ There are few studies on chromium LPT and these were performed with various protocols and scoring methods, leading to inconsistent results and doubt about its clinical applicability.¹⁴ The aim of this study was to determine the appropriate salt, concentration and period of incubation for chromium LPT assay.

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Conflict of interest: none declared.

Accepted for publication 19 September 2007

Methods

The study was approved by the local ethics committee, and informed consent was obtained from all participants.

Patients and controls

In total, 20 male patients (mean age 45 years, range 28–63), having eczematous lesions and patch testing consistent with chromium allergy, were recruited in our clinic. Another group of 20 male patients (mean age 51 years, range 26–81) without history of chromium allergy and with negative patch testing to potassium dichromate were used as the control group.

Patch testing

Patients and controls were patch tested using the Brazilian standard patch testing series (FDA Allergenic®; Rio de Janeiro, Brazil),⁴ which comprises 30 antigens, including 0.5% potassium dichromate in petrolatum. The allergens were applied under Finn chambers for 2 days on sticky tape (3M®; St Paul, MN, USA) over the upper back. Results were read at 48 and 96 h, and the reactions were scored as follows: + = erythema and infiltration, ++ = erythema, infiltration and vesicles and +++ = erythema, infiltration and confluent vesicles. Erythema and infiltration were the minimum criteria for considering a patch test positive.

Chromium-specific lymphocyte proliferation assay

Mononuclear cells were isolated from heparinized venous blood by centrifugation on a density gradient (Ficoll-Hypaque). They were then washed and resuspended (2×10^6 cells/mL) in RPMI 1640 medium supplemented with gentamicin and 10% pooled normal human AB serum. Aliquots (100 µL) of cell suspension were cultivated in the presence of either 100 µL of RPMI 1640 medium (nonstimulated cultures) or 100 µL of one of several chromium solutions prepared in RPMI 1640 medium (stimulated cultures). Cells were also stimulated with 2.5 µg/mL of phytohaemagglutinin (Gibco; Invitrogen, Carlsbad, CA, USA) and 5 µg/mL of pokeweed (*Phytolacca americana*; Sigma, St Louis, MO, USA) as positive controls. Cultures were performed in triplicate in 96-well flat-bottomed plates (TPP, Switzerland) for 3 and 6 days, as described previously.¹⁵ Eighteen hours before the cell harvesting, 1 µCi of radiolabelled thymidine (Amersham Pharmacia Biotech, Amersham, Buckinghamshire, UK) was added to each culture. T-cell proliferation was measured by ³H-thymidine incorporation detected by a scintillation counter (Betaplate, Wallac, Finland). The stimulation index (SI), calculated as the ratio between the mean of ³H-thymidine

uptake in stimulated and nonstimulated cultures, was used to determine the lymphoproliferative response ($SI = \text{average } ^3\text{H-thymidine count in stimulated culture} / \text{average } ^3\text{H-thymidine count in nonstimulated culture}$). Reactivity was considered positive at $SI > 3.0$.

Tested substances and concentrations

Trivalent ($\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$; purity $\geq 96\%$) and hexavalent ($\text{K}_2\text{Cr}_2\text{O}_7$; purity $\geq 99.9\%$) chromium compounds (Merck, Darmstadt, Germany) were used. Stock solutions were prepared in RPMI 1640 medium and half the volume was filtered (0.22 µm). All aliquots were stocked at -70°C until use.

In a preliminary study, 3- and 6-day cultures from six chromium-sensitized patients were stimulated with a broad range of serial dilutions (10^{-3} – 10^{-9} mol/L) of filtered and nonfiltered Cr(III) and Cr(VI) to establish the parameters that should be investigated further.

No response was seen with 3-day cultures but three patients responded ($SI > 3$) to 6-day cultures: all of them to 10^{-3} mol/L nonfiltered Cr(III) solution and 2 – 10^{-3} mol/L filtered Cr(III) solution, two to different, noncontiguous filtered Cr(VI) solutions, and none to nonfiltered Cr(VI) solutions.

Therefore, for the definitive study, 6-day cultures were tested with 7.5×10^{-3} , 5×10^{-3} , 2.5×10^{-3} , 10^{-3} , 7.5×10^{-4} , 5×10^{-4} , 2.5×10^{-4} and 10^{-4} mol/L of filtered and nonfiltered Cr(III) solutions. The final culture chromium concentration was half of the solution added.

Statistical analysis

The nonparametric Mann–Whitney *U*-test (GraphPad Prism® software, version 3.0; GraphPad Software, San Diego, CA, USA) was used to compare SI values obtained with patients and controls at each chromium concentration. Significance was set at $P < 0.05$.

Results

Patch testing

All patients and none of the control group had positive patch tests to potassium dichromate. There were 11 patients who were also positive to carba mix and seven to thiuram mix. Five controls were positive to neomycin sulphate, four to ethylenediamine dihydrochloride, three to carba mix, quaternium-15 and nickel sulphate, and two to cobalt chlorate and thiuram.

Definitive study

In general, the new range of chromium concentrations stimulated the patients lymphocytes at some degree, with SIs being higher than those found in the control group for most of the tested solutions ($P < 0.05$) (Table 1). Under our experimental conditions, the highest (7.5×10^{-3} mol/L) and the lowest ($10^{-4} - 5 \times 10^{-4}$ mol/L) dilutions of chromium were not able to induce good proliferation.

The best results were obtained using 7.5×10^{-4} , 10^{-3} , 2.5×10^{-3} and 5×10^{-3} mol/L of chromium compounds. Taking all SI > 3 results of filtered Cr(III) stimulation within this range, eight patients and two controls responded to these concentrations. Seven of these and none of the control group responded to 10^{-3} mol/L solution (Figs 1,2). With the same concentrations of nonfiltered Cr(III) solutions, 13 patients and only one control showed SI > 3 (Figs 3,4). For the total subject cohort, the highest response was observed with 2.5×10^{-3} mol/L of nonfiltered Cr (III), to which 12 patients and one control had SI > 3.

Sensitivity and specificity were calculated using various cut-offs (Table 1). The best ratios were detected using filtered and nonfiltered solutions from 7.5×10^{-4} mol/L up to 5×10^{-3} mol/L. In this range,

Table 1 Sensitivity and specificity of filtered and non-filtered Cr(III) solutions.

Concentration (mol/L)	Stimulation index					
	>2		>2.5		>3	
	Se (%)	Sp (%)	Se (%)	Sp (%)	Se (%)	Sp (%)
Filtered						
10^{-4}	5	100	5	100	5	100
2.5×10^{-4} *	10	100	0	100	0	100
5×10^{-4} *	30	100	30	100	30	100
7.5×10^{-4} *	40	90	35	95	30	95
10^{-3} *	55	95	40	100	35	100
2.5×10^{-3} *	35	95	25	95	25	95
5×10^{-3} *	40	100	30	100	20	100
7.5×10^{-3} *	15	100	5	100	0	100
Non-filtered						
10^{-4}	10	100	0	100	0	100
2.5×10^{-4} *	5	100	5	100	5	100
5×10^{-4} *	40	100	25	100	20	100
7.5×10^{-4} *	45	85	35	90	35	95
10^{-3} *	60	75	50	85	45	95
2.5×10^{-3} *	75	80	60	85	60	95
5×10^{-3} *	55	85	35	95	20	100
7.5×10^{-3}	15	100	5	100	5	100

Se, sensitivity; Sp, specificity. * $p < 0.05$.

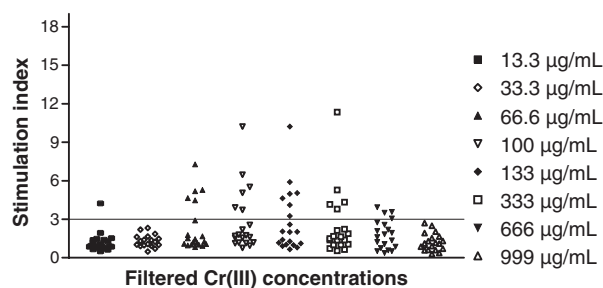


Figure 1 Stimulation indexes found in patients challenged with filtered Cr(III).

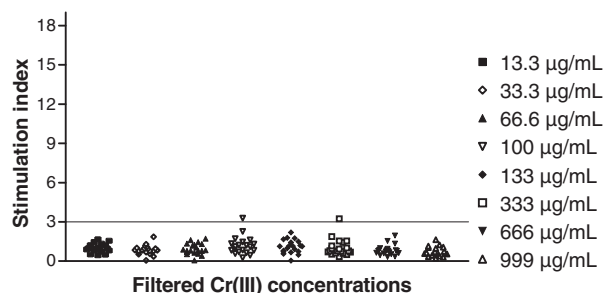


Figure 2 Stimulation indexes found in controls challenged with filtered Cr(III).

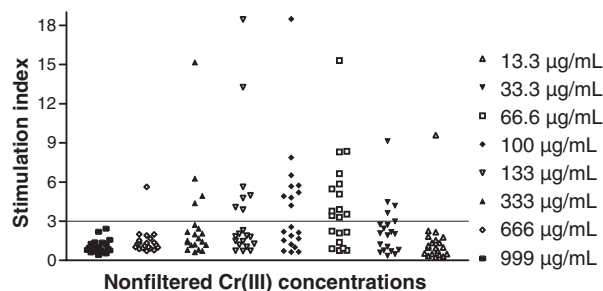


Figure 3 Stimulation indexes found in patients challenged with nonfiltered Cr(III).

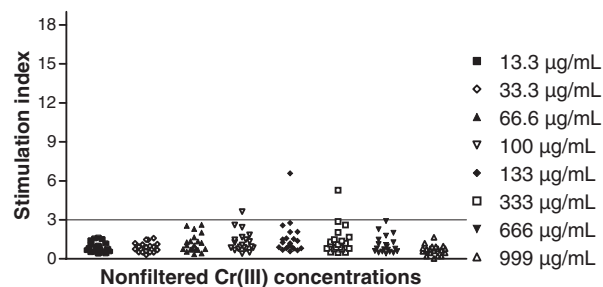


Figure 4 Stimulation indexes found in controls challenged with nonfiltered Cr(III).

filtered and nonfiltered chromium solutions had an accuracy of 65% and 80%, respectively.

Lymphocytes from all patients and controls responded to phytohaemagglutinin and pokeweed, and no contamination was detected.

Discussion

Although patch testing is routinely used to diagnose ACD, there is a need for another confirmatory test. LPT may be this alternative. In this study we investigated a number of LPT parameters to optimize testing for the diagnosis of chromium ACD. Once responses with various chromium salts, concentrations and incubation periods were obtained, we focused on these parameters in a preliminary study to investigate the best conditions in which to perform chromium LPT.^{14,16–20} Initially, we chose a broad range of concentrations of chromium chloride [Cr(III)] and potassium dichromate [Cr(VI)] to be tested with both short and long incubation periods. We also evaluated the role of solution filtering, a process that can be used to reduce the possibility of culture contamination and to eliminate any residual particles.

After the preliminary study, we were convinced that 3-day cultures were not appropriate, as there had been no response with these. The 6-day cultures were more promising, as three patients tested with Cr(III) solutions showed lymphoproliferative response (SI > 3). Responses to 6-day cultures with potassium dichromate did not seem to be as consistent as those found with Cr(III), as only two patients responded, both only with filtered solutions and in noncontiguous solutions (one to 10^{-6} and 10^{-7} mol/L and another to 10^{-9} mol/L). In addition, both patients had also responded to Cr(III) solutions. We therefore decided not to use Cr(III) or Cr(VI) 3-day cultures and Cr(VI) 6-day cultures. The results also indicated that more Cr(III) concentrations around 10^{-3} mol/L should be tested to determine the optimum concentration range of this salt in 6-day cultures.

Before this study, only AL-Tawil *et al.*¹⁹ had compared short vs. long-term cultures (Table 2). They found that periods of ≥ 5 days are appropriate. However, Carando *et al.*²⁰ obtained strong results stimulating 56-h cultures of two patients sensitized to chromium-containing prostheses. Nevertheless, our results confirm that longer periods are necessary. This was unsurprising; antigen stimulation initially activates only those T cells presenting the specific antigen receptor, and this is followed by activation of additional cells. Thus, a longer culture period of 6–7 days has been the standard approach.²¹

With regard to choice of salt, the preliminary study indicated that the stimulation with chromium chloride could be superior to using potassium dichromate. AL-Tawil *et al.*¹⁹ found that patients who respond to potassium dichromate also respond to chromium chloride, in agreement with our findings, but they did not clarify if the reverse is true. Interestingly, these researchers used only chromium chloride to stimulate cells from patient with chromium allergy in their later studies.^{22,23} In addition, in the study of Räsänen *et al.*,¹⁴ five of six patients who responded to this test had higher SI values with chromium chloride than with two hexavalent compounds, including potassium dichromate. Thus, despite the lack of clear statistical evidence, we believe that chromium chloride is at least as efficient as potassium dichromate to stimulate cultures from chromium-sensitized patients. This is expected, as Cr(III) is believed to be the actual chromium hapten.¹³

To compare the concentrations of chromium chloride used in our study with those of previous assays, all values were converted to the same unit ($\mu\text{g CrCl}_3/\text{mL}$) (Table 2). Christiansen *et al.*¹⁸ and AL-Tawil *et al.*¹⁹ found the best final culture concentrations to be between 25 and 100 $\mu\text{g}/\text{mL}$ of Cr(III). In our second experiment (definitive study), we tested patients with concentrations from 13.3 to 999 $\mu\text{g}/\text{mL}$. The highest sensitivity and specificity values were found with 100–666 $\mu\text{g}/\text{mL}$. Although our ideal range of testing

Table 2. Previous assays that used CrCl_3 to stimulate patient lymphocytes.

Authors	Patients (n)	Controls (n)	CrCl_3 concentrations tested (mg/mL)	Best CrCl_3 concentrations (mg/mL)	Period of culture tested (days)	Se (%)	Sp (%)
Christiansen <i>et al.</i> ¹⁸	24	11	0.26–266	26.65	6	62.5	91
Al-Tawil <i>et al.</i> ¹⁹	31	24	6.25–200	25, 50 and 100	3–8	65	92
Carando <i>et al.</i> ²⁰	2	–	0.5	0.5	2.33 (56 h)	100	
Räsänen <i>et al.</i> ¹⁴	8	8	6.25–50	25 and 50	6–8	75	100
Martins <i>et al.</i>	20	20	13.3–999	100, 133, 333 and 666	3 and 6	65	95

Se, sensitivity; Sp, specificity.

concentrations was different to those of the previous studies., our values of sensitivity and specificity were similar to those found by Christiansen *et al.* (sensitivity of 62.5% and specificity of 91%) and Al-Tawil *et al.* (sensitivity of 65% and specificity of 92%).

There is no standard criterion in chromium LPT to consider a reaction as positive. Previous studies have used $SI > 1$ and greater than the SI of a simultaneously tested control¹⁹, $SI > 2.5$ ¹⁴ or $SI > 3$.¹⁸ We calculated the sensitivity and specificity of LPT using various cut-offs, from one to three, and chose $SI > 3$ to better discriminate patients with chromium allergy from other subjects. $SI > 2$ or > 2.5 would compromise the specificity and would not greatly improve the sensitivity of the test.

Studying the importance of the filtration process, we found that the same molar solutions generated different SIs, depending on whether they were filtered or not, affecting the sensitivity of the test. This fact may be due to the presence of small particles that could have remained in the nonfiltered solutions, despite the care taken with homogenization. Because no contamination was detected and better sensitivity was obtained with nonfiltered solutions, we believe that chromium chloride solutions do not need to be filtered.

This is the first report to test chromium LPT specificity using a control group sensitized to diverse antigens. One control, who was sensitized to quaternium-15, had $SI > 3.0$. Another two controls and one patient were also sensitized to quaternium-15; all of them had $SI < 3.0$. The false-positive control was patch tested again and only a slight erythema (no infiltration or vesicles) was noted at 48 and 96 h, confirming that the patient was not sensitized to potassium dichromate. A new LPT was negative ($SI < 3.0$). A false-negative LPT case was also tested again; both patch testing and LPT maintained the previous results.

The main limitation faced with this method is its low sensitivity. This may be due to: (i) use of monocytes instead of Langerhans' cells as antigen-presenting cells; (ii) low sensitivity of the method itself; and/or (iii) need for a booster to start or increase the reaction *in vitro*. Räsänen *et al.*¹⁴ worked with Langerhans' cells and achieved better results compared with those found with monocytes. Unfortunately, they tested only four patients and had no control. Trattner *et al.*²⁴ measured lymphocyte activation by interferon (IFN)- γ release. Although IFN- γ was significantly higher in patients with contact allergy to potassium dichromate than in controls, the sensitivity and specificity were low, at 73.7% and 71.42%, respectively, inferior to the results we found with 2.5×10^{-3} mol/L nonfiltered solutions using a SI cut-off of

2.0. Rustemeyer *et al.*²⁵ and Moed *et al.*⁶ showed that cytokines added to the cultures increase the proliferation response and cytokine production by allergen-specific T cells. However, only seven chromium-sensitive patients and controls were tested with this method.⁶

In conclusion, we recommend the use of nonfiltered chromium chloride solutions in the range 7.5×10^{-4} to 5×10^{-3} mol/L to stimulate 6-day cultures to test for chromium ACD by LPT. In an attempt to improve the testing sensitivity, we are working with cultures stimulated with cytokines and comparing two detection methods.

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