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Lymphocyte Stimulation by Trivalent and Hexavalent Chromium Compounds in Patients with Chromium Sensitivity

An Aid to Diagnosis

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Peripheral blood lymphocytes from 31 patients with a positive patch test to potassium dichromate ($K_2Cr_2O_7$) and from 24 healthy controls were stimulated with various concentrations of chromium chloride ($CrCl_3$) and/or chromium basic sulphate ($Cr_4(SO_4)_5(OH)_2$), sodium chromate (Na_2CrO_4) or $K_2Cr_2O_7$ on various days of culture. Both trivalent and hexavalent chromium compounds could induce lymphocyte transformation, as measured by increased DNA synthesis. The response occurred in the T-enriched population and was monocyte dependent. Lymphocytes from 11 of these patients could not be stimulated with the chromium compounds in vitro, whereas the in vivo serial dilution test (SDT) was positive in 4 and negative in 7 of them. Lymphocytes from 2 patients with a negative in vivo SDT showed a positive response in vitro. The strength of the in vivo SDT results did not correlate well with the height of in vitro responses. The DNA synthesis test seems to be a reliable in vitro method to aid in the diagnosis of chromium sensitivity. *Key words: Chromium sensitivity; Lymphocyte stimulation; In vitro criteria.* (Received November 16, 1982.)

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Chromium eczema is one of the most serious occupational dermatoses (26) and in industrialized countries chromate is the most common sensitizer in males (3, 6). It was suggested by some authors (21, 29, 30) that only hexavalent chromium compounds were sensitizers while others (12, 13, 14, 23) have demonstrated positive patch test results with trivalent chromium compounds. Although patch testing is done to confirm the clinical impression, its results can sometimes be misleading being false-positive or false-negative (7, 10). Thus we tried to ascertain wether trivalent or hexavalent chromium compounds could stimulate lymphocytes in vitro, as measured by the DNA synthesis test; and if so, whether this test could be used to assist the diagnosis of chromium sensitivity.

MATERIAL AND METHODS

Patients. Thirty-one patients with a positive patch test (++ or more) to $K_2Cr_2O_7$ were included in this study. They ranged in age from 27 to 79 years. Four patients with a positive patch test to $NiSO_4$ and/or $CoCl_2$ were also included to test for specificity. Their ages ranged from 42 to 50 years.

Controls. Lymphocytes from altogether 24 age- and sex-matched healthy individuals with neither eczema nor a case history of metal contact allergy were included in the experiments. Cord blood lymphocytes from two newborn infants were also tested.

Patch testing was performed according to Pirilä (25). The conditions, time of exposure and reading of the test were as previously described (1). None of the patients had had active eczema for at least 2 weeks prior to testing.

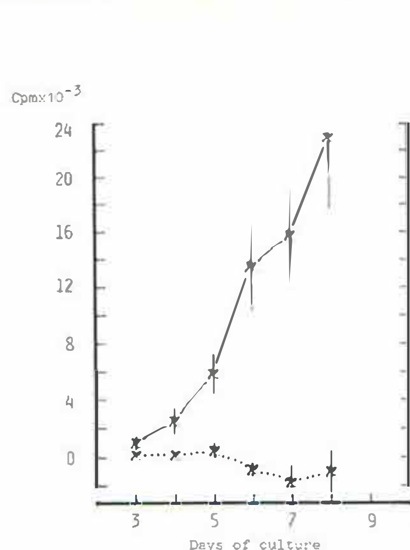

Fig. 1. Time response curve for peripheral blood lymphocytes from 20 patients with a positive patch test to $K_2Cr_2O_7$ (×—×) and 19 controls (×---×) using 50 μg $CrCl_3$/ml culture. The mean increment counts per minute and standard error values are plotted.

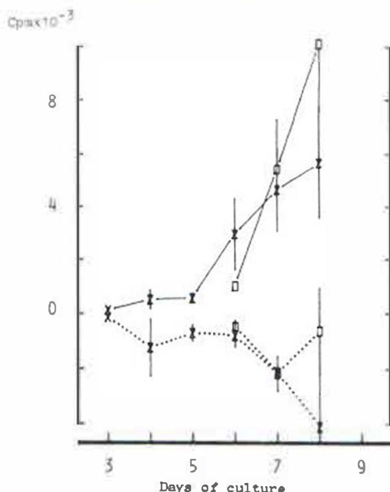

Fig. 2. Stimulation of lymphocytes from 11 patients with a positive patch test to $K_2Cr_2O_7$ (×—×) and 9 controls (×---×) by 0.1 μg Na_2CrO_4/ml. Lymphocytes from 5 of these patients (O—O) and 3 controls (O· · ·O) were also stimulated by 0.1 μg $K_2Cr_2O_7$/ml. The mean increment counts per minute and standard error values are plotted.

In vivo serial dilution test (SDT) was performed using the following concentrations of $K_2Cr_2O_7$ in distilled water: 0.25, 0.125, 0.0625, 0.0312, 0.0156, 0.0078 and 0.0039%. A ++ reaction or more with any of these concentrations was considered as positive.

Preparation of chromium compounds and PHA. Stock solutions of 1% of two trivalent compounds: $CrCl_3 \cdot 6H_2O$ (BDH Chemicals Ltd, Poole, England) and $Cr_4(SO_4)_5(OH)_2$ (Fluka AG, Buchs, Switzerland), and two hexavalent compounds: $K_2Cr_2O_7$ (Merck, Darmstadt, Germany) and $Na_2CrO_4 \cdot 4H_2O$ (Mallinckrodt, St Louis, Mo., USA) were prepared in distilled water. Trivalent chromium compounds were prepared in modified Eagle's minimum essential medium (MEM) (Flow Laboratories, Irvine, Scotland) without serum, giving final concentrations in culture of 6.25, 12.5, 25, 50, 100 and 200 μg/ml. Hexavalent chromium compounds were added to give final concentrations of 0.025, 0.05, 0.1, 0.2, 0.4 and 0.8 μg/ml. Phytohaemagglutinin (PHA) (Wellcome) was added to a final concentration of 50 μg/ml as a positive control.

Mononuclear cell preparation and assay of DNA synthesis. This was performed as described perviously (1). Monocytes were isolated by adherence to plastic and more than 90% of these cells ingested Candida. The non-adherent cell suspension was iron-treated and then fractionated into T- and B-enriched lymphocyte populations (24). Approximately 60% of the B-enriched population showed positive surface membrane immunofluorescence and 5% were E-rosette positive. The T-enriched population contained more than 80% E-rosette positive cells and less than 5% Ig-positive cells.

Statistical method. Student's *t*-test was used. P-values<0.05 were considered significant.

RESULTS

All the 31 patients showed a ++ reaction or more when patch tested with 0.5% $K_2Cr_2O_7$. However, the in vivo SDT was negative in 9 of the 30 patients who agreed to participate.

Lymphocytes from 20 patients (excluding 11 patients, see below) gave their highest response on day 7 of culture when stimulated with 6.25, 12.5 and 200 μg $CrCl_3$/ml and on day 8 when stimulated with 25, 50 and 100 μg $CrCl_3$/ml while lymphocytes of controls

showed either a negative or a weak response (Fig. 1). Response of the patients' lymphocytes were significantly higher than those of the controls when stimulated with 25, 50 and 100 μg $CrCl_3$/ml on days 5–8 of culture ($P<0.001$) and also when stimulated with 6.25 μg on days 5 and 8, 12.5 μg on days 5–8 and 200 μg on days 3–8 of culture ($P<0.05$). Responses of lymphocytes from individual patients were almost always higher than those of the controls tested simultaneously with any of these concentrations on days 3–8 of culture.

Lymphocytes from some of these patients were challenged with another trivalent chromium compound, $Cr_4(SO_4)_5(OH)_2$ for various days in culture. The cells responded in a similar manner as when challenged by $CrCl_3$ (Table I).

Lymphocyte responses to stimulation with two hexavalent chromium compounds, $K_2Cr_2O_7$ and Na_2CrO_4, are exemplified in Fig. 2. The patient lymphocyte responses to either of these compounds were higher ($P<0.05$) than the responses of control lymphocytes, except on days 3 and 4 of culture. Similar figures and levels of significance were obtained when lymphocytes were stimulated with 0.2 μg and 0.4 μg/ml culture. Concentrations higher than 0.8 μg/ml were toxic. Patient lymphocytes that responded to stimulation by hexavalent chromium compounds were also responders to $CrCl_3$.

Cord blood lymphocytes from 2 newborn infants were then stimulated with various concentrations of $CrCl_3$, $K_2Cr_2O_7$ and Na_2CrO_4 for 3–8 days in culture. Unlike $NiSO_4$, which was included as a positive control (1), these chromium compounds could not induce cord blood lymphocyte proliferation (data not shown).

From the above data it was possible to identify certain criteria that should be fulfilled in order to define a positive response in vitro. Thus, reactivity to chromium should be considered as positive in a patient whose lymphocytes, when stimulated with 25, 50 and 100 μg $CrCl_3$ or $Cr_4(SO_4)_5(OH)_2$/ml culture, give stimulation indices of more than one and

Table I. *In vitro lymphocyte responses (SI) to various trivalent chromium compounds*

Expt. no.	Pat. no.	Days	Background (cpm)	$Cr_4(SO_4)_5(OH)_2$ (μg/ml)			Background (cpm)	$CrCl_3$ (μg/ml)		
				25	50	100		25	50	100
I	26	5	2 146	2.9	2.3	6.0	1 130	5.3	13.8	13.9
		6	2 476	3.2	3.4	9.8	2 095	5.9	18.9	15.9
		7	1 415	11.3	12.9	45.4	1 037	6.1	3.5	47.9
		8	2 298	13.2	9.9	21.1	1 919	23.5	39.6	46.0
	C[a]	5	2 161	0.7	0.7	0.6	1 871	0.9	2.7	1.5
		6	4 592	0.3	0.3	1.4	3 764	1.0	1.1	0.9
		7	5 925	1.0	0.3	0.7	3 032	0.5	1.0	1.1
		8	2 372	1.5	1.3	2.1	3 701	1.4	2.1	2.5
II	60	7	4 095	10.6	13.0	20.6	4 895	7.8	13.4	13.5
		8	4 932	6.7	9.0	13.6	3 938	7.5	12.5	18.9
		9	4 486	7.2	7.3	8.3	3 705	8.9	11.2	14.4
	61	7	4 968	3.9	4.6	6.2	3 046	7.1	11.9	10.5
		8	5 571	4.4	4.6	6.9	5 089	5.7	6.2	10.2
		9	5 446	4.7	5.4	9.6	3 419	7.1	9.0	13.1
	62	7	1 529	3.1	10.8	6.8	1 051	16.2	14.9	9.4
		8	1 446	7.2	23.3	12.9	1 553	10.3	11.6	4.8
		9	1 848	6.1	17.2	10.0	1 629	18.0	6.9	2.0
	C	7	2 658	0.5	0.3	0.6	2 206	0.6	0.6	0.4
		8	3 057	0.6	0.6	1.1	3 003	0.4	0.4	0.4
		9	5 000	0.5	0.2	0.4	4 039	0.3	0.5	0.2

[a] C = control.

Table II. *Specificity of lymphocyte responses in vitro*

Cells from patients with indicated metal allergies were tested with $CrCl_3$ and SI on day 6 of culture are shown

Expt. no.	Pat. no.	Back-ground (cpm)	Concentration of $CrCl_3$ (μg/ml)						Clinical metal allergy	Evaluation of chromium sensitivity in vitro
			6.25	12.5	25	50	100	200		
I	2	1 367	1.3	1.1	1.3	1.3	1.8	1.2	Cr	+
	4	960	1.7	1.2	1.7	1.1	2.9	1.9	Cr Co	+
	BK	4 222	0.6	0.4	0.5	0.7	0.6	0.5	Ni Co	–
	C[a]	2 213	0.7	0.8	0.9	0.6	0.9	0.6	–	–
	C	835	0.7	0.6	0.6	0.7	1.6	1.1	–	–
II	52	2 820	4.8	5.0	6.9	7.4	9.6	8.2	Cr Co Ni	+
	64	1 404	3.4	6.9	11.3	15.2	11.9	11.7	Cr	+
	GE	319	1.2	0.9	0.8	1.1	1.0	0.5	Co	–
	ML	1 547	0.6	0.6	0.6	0.6	0.4	0.2	Ni	–
	C	386	0.6	1.0	1.2	0.8	0.4	0.6	–	–
III	SF	999	nd[b]	0.8	0.8	0.6	×0.7	nd	Ni	–
	49	4 331	nd	4.0	4.7	7.2	9.3	nd	Cr Co Ni	+
	C	3 318	nd	0.4	0.4	0.3	0.2	nd	–	–
	C	778	nd	0.8	0.9	0.7	0.2	nd	–	–

[a] C = control.
[b] nd = not done.

greater than those of the control lymphocytes simultaneously tested, on days 6, 7 and 8 of culture. These conditions also apply when the hexavalent chromium compounds $K_2Cr_2O_7$ and Na_2CrO_4 were used at final concentrations of 0.1, 0.2 and 0.4 μg/ml.

To test the specificity of chromium compound-induced activation, lymphocytes from 4 patients with a positive patch test to $NiSO_4$ and/or $CoCl_2$ were stimulated with various concentrations of $CrCl_3$ for various days in culture. The response of lymphocytes from all the patients with a positive patch test to $NiSO_4$ and/or $CoCl_2$ failed to fulfil the above-mentioned criteria, thus confirming the specificity of the stimulation (Table II).

Lymphocytes of 2 of the 9 patients who had a negative response to serial dilutions of $K_2Cr_2O_7$ in vivo responded to stimulation with $CrCl_3$ in vitro and were included in the time–response curves, while lymphocytes of the remaining 7 patients could not be stimu-

Table III. *Skin and lymphocyte responses of 31 patients with a positive patch test to 0.5% $K_2Cr_2O_7$ and 24 controls*

Patch test (0.5% $K_2Cr_2O_7$)	Serial dilution test	DNA synthesis test	No. of patients	No. of controls
+	+	+	17	
+	–	–	7	
+	+	–	4	
+	–	+	2	
+	nd[a]	+	1	
nd	nd	–		24

[a] nd = not done.

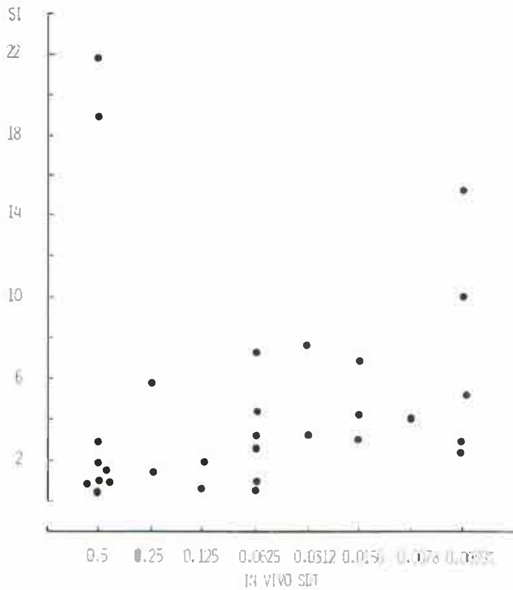

Fig. 3. Absence of correlation between the strength of patient's responses to in vivo serial dilutions of $K_2Cr_2O_7$ and the height of in vitro lymphocyte responses to stimulation by 50 μg $CrCl_3$/ml on day 6 of culture.

lated in vitro. On the other hand, lymphocytes of 4 other patients who had a positive response to the SDT in vivo did not show a positive response in vitro. Responses of lymphocytes from the latter 11 patients were thus excluded from the time–response curves (see above). These results were confirmed by retesting lymphocytes, taken on different occasions from 2 patients of each of the three groups. The patients' skin and lymphocyte responses are summarized in Table III.

We were unable to find a good correlation between the strength of in vivo SDT results and in vitro lymphocyte responses (Fig. 3) to stimulation by 25, 50 and 100 μg$CrCl_3$/ml on days 6, 7 and 8 of culture.

Table IV. *[³H]-thymidine uptake (cpm) by mononuclear cells of a patient with chromium sensitivity and a control following stimulation by 50 μg $CrCl_3$/ml on day 6 of culture*

The response occurred in the T-enriched population and was monocyte-dependent

Pat. no.	Addition		Cell suspension[a]		
	M[b]	$CrCl_3$	Unfractionated	T-enriched	B-enriched
6	–	–	845	325	472
	–	+	31 114	1 581	1 039
	+	–		430	995
	+	+		9 088	671
C[c]	–	–	3 840	408	2 13[illegible]
	–	+	2 250	978	858
	+	–		1 511	961
	+	+		1 440	2 191

[a] 1×10^5 cells /well.
[b] 5×10^3 monocytes/well.
[c] C = control.

Peripheral blood mononuclear cells from a few patients with chromium sensitivity and controls were fractionated into T- and B-enriched monocyte-depleted populations which were then stimulated with 50 µg $CrCl_3$/ml for 6 days in culture with or without the addition of autologous monocytes. Proliferation occurred in the T-enriched fraction and was dependent on the presence of monocytes. The results of one experiment are shown in Table IV.

Lymphocytes from all patients and controls responded to PHA.

DISCUSSION

Jung (19) and Lischka (20) have shown that lymphocytes from chromium-sensitive patients could be induced to transform into blasts following the addition of $K_2Cr_2O_7$, but they gave no criteria for in vitro reactivity, nor did they use other chromium compounds. Earlier, Grosfeld et al. (18) found unconvincing positive and negative results following stimulation of patients' lymphocytes by $K_2Cr_2O_7$. From the results of our study, using both trivalent and hexavalent chromium compounds, we could define certain criteria for in vitro reactivity.

Chromium compounds of both valencies have been shown to provoke a positive patch test reaction when applied to the skin of chromium-sensitive patients, trivalent chromium being used at a much higher concentration than hexavalent chromium (12) and the latter reduced to the former by certain constituents of the skin (31). The true nature of the antigen is not yet fully known, but it has been suggested that chromium in its trivalent form, bound to relevant proteins, forms the full antigen (27).

Both trivalent (Fig. 1, Table I) and hexavalent (Fig. 2) chromium compounds, when added to culture, could induce proliferation of peripheral blood lymphocytes from chromium-sensitive patients. The concentration of trivalent chromium required for optimal stimulation was much higher than that of hexavalent chromium. This can be explained by the findings that trivalent chromium binds strongly to proteins (17, 28) most of which, if irrelevant, might be converted into a non-immunogenic form. In culture, most of the hexavalent chromium enters the cell and is then reduced to the trivalent form (17). Hexavalent chromium is also reduced to trivalent chromium by components of culture medium (28). Thus the proposition that trivalent (and not hexavalent) chromium is the sensitizing agent cannot be ruled out and requires further experimentation.

The $CrCl_3$-induced proliferation of lymphocytes from patients sensitive to $K_2Cr_2O_7$ (but not from those sensitive to $NiSO_4$ and/or $CoCl_2$) demonstrates the specificity of activation. Some authors (8, 15) have shown that co-existing allergy to chromium, nickel and cobalt is not due to cross-sensitization, but to the occurrence of these metals in sensitizing objects to which the patients are exposed.

The purpose of patch testing is to discover a contact allergy and if the reaction elicited using the standard concentration is suspected to be of irritant type, the testing should be repeated with the substance diluted (11). An allergic reaction to an allergen applied to the skin of a sensitive individual gradually diminishes in severity the weaker the test solution, whereas a toxic reaction stops abruptly below a certain concentration (2, 4). In our in vitro study this does not seem to apply, since lymphocytes from 2 patients who gave a positive skin reaction only to the 0.5% concentration of $K_2Cr_2O_7$, showed the highest responses (Fig. 3), whereas other patients, who were patch test positive both to the standard concentration and to some or all the dilutions, showed weaker or even negative lymphocyte responses. There does not seem to be a good correlation between the strength of the in vivo SDT results and the height of in vitro lymphocyte responses. This only indicates

that other mechanisms, in addition to those caused by proliferating T lymphocytes, play a role in the development of a positive patch test reaction (16, 32).

Lymphocytes from 11 of the 31 patients showed a negative response in vitro. The in vivo SDT was negative in 7 of these patients, but positive in 1–3 dilution steps in the remaining 4 patients. Fisher (9) recommended that for routine patch testing for chromium dermatitis a 0.25% aqueous solution of $K_2Cr_2O_7$ should be used, since a concentration of 0.5% may act as a primary irritant. Taking other possible causes of false-positive patch test reactions into consideration (5, 22), a probable explanation for the positive in vivo SDT in the 4 patients is hyperirritability of the patients' skin. Moreover, the negative in vitro responses in 2 of the latter patients may be explained by the fact that patch testing was performed 6 and 12 years ago respectively and that they might have lost their reactivity during that period. Thormann et al. (33) reported that 10 of 48 patients who had had a positive patch test response to 0.5% $K_2Cr_2O_7$ had lost their positive reactivity when retested 4–7 years later.

Although some workers (28, 34, 35) have used the leukocyte migration inhibition test to demonstrate chromium sensitivty, we consider that the DNA synthesis test is less complicated and is a reliable method which can be used to assist in the diagnosis of chromium sensitivity and by which false-positive patch test reactions could be detected. We have shown that the response of lymphocytes to stimulation by $CrCl_3$ occurred in the T cell population and was monocyte dependent. We intend to ascertain whether this response is HLA restricted and whether the same (or a different) subpopulation of lymphocytes is triggered when stimulated by trivalent and hexavalent chromium compounds.

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