

Lack of antagonism to Ni^{2+} and Co^{2+} contact allergy from other essential divalent metal ions

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The potential antagonistic effects of Ca^{2+} , Cu^{2+} , Fe^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} on contact allergy to Co^{2+} and Ni^{2+} were studied. The immune response was characterized by the Co^{2+} or Ni^{2+} mediated cellular [methyl- ^3H]thymidine uptake in peripheral blood mononuclear cell (PBMC) cultures from 6 subjects contact-allergic to Co^{2+} and Ni^{2+} and 6 non-allergic control individuals. Results from the in vitro experiments were further evaluated with Co^{2+} -sensitized guinea pigs according to the modified Freund's complete adjuvant test. Ni^{2+} and Co^{2+} (10–50 μM) significantly increased the lymphocyte proliferation in PBMC cultures from contact-allergic subjects in comparison with those from control individuals. Pretreatment of the PBMCs with Ca^{2+} , Fe^{2+} , Mg^{2+} (10–100 μM) or Mn^{2+} (1–10 μM) did not influence, while Zn^{2+} (100 μM) enhanced, and Cu^{2+} (5 and 10 μM) markedly reduced the Ni^{2+} and Co^{2+} mediated cellular [methyl- ^3H]thymidine uptake. The inhibition of the Ni^{2+} - and Co^{2+} -induced cell proliferation by Cu^{2+} in vitro was probably related to toxicity, since the viability of the cells was significantly reduced by applied combinations of Ni^{2+} or Co^{2+} with Cu^{2+} . Topical pretreatment of Co^{2+} -sensitized guinea pigs with maximum non-irritating doses of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.8%) did not affect the challenge testing to $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.1 and 0.3%). In conclusion, our combined in vitro and in vivo results indicate that Ca^{2+} , Cu^{2+} , Fe^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} are not able to antagonise the formation of Ni^{2+} and Co^{2+} antigens.

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Ni^{2+} and Co^{2+} are 2 of the most frequent causes of contact allergy (1). The antigen-specific T lymphocytes mainly belong to the CD4^+ subpopulation (2, 3), but to a lesser frequency CD8^+ expression has also been found (4). T cell activation is HLA class II restricted, for Ni^{2+} most often associated with HLA-DR molecules (2, 3, 5). Peripheral blood T lymphocytes and antigen-specific T cell clones from contact-allergic individuals can be stimulated by low concentrations of Ni^{2+} and Co^{2+} , and show a cytokine secretion corresponding with activated Th1 cells (6, 7). This in vitro antigen-induced lymphocyte proliferation is an appropriate model for mechanistic studies on contact allergy (8). The antigenic structures formed by the metal haptens and the epitopes recognized by the metal-ion-reactive T cells are essentially unknown.

Ni^{2+} and Co^{2+} allergy is believed to be initiated

by the formation of specific coordination complexes with electron-rich residues of proteins (or other biomolecules) (9). The mechanism of "foreign" protein formation is probably strongly influenced by the nature of the metal ion chelating ligands, since they largely determine the coordination and stability of the complexes. Histidine and cysteine residues are likely candidates to be involved in the metal ion – protein interactions, since Ni^{2+} and Co^{2+} have a high affinity for both ligands, with which they can form complexes with varying geometries like, e.g., tetrahedral, square planar and octahedral structural arrangements (10, 11). However, divalent metal ions can share physicochemical properties with other metal ions, such as ionic radii, geometric arrangement of complexes and affinities for biological ligands (12). For example, after substitution of the catalytic metal ion, metal-

loenzymes may still yield enzymatically active species (12–14). From those apparent similarities in binding properties, it could be expected that other divalent metal ions compete for binding sites for Ni^{2+} and Co^{2+} and as such antagonize the formation of antigenic coordination complexes. Indications of potential antagonism of Ni^{2+} induced biological effects by other divalent metal ions have been obtained for e.g. Zn^{2+} , Ca^{2+} and Mg^{2+} in different experimental systems (15–17). Moreover, in Ni^{2+} -allergic individuals, Mg^{2+} , Mn^{2+} and Zn^{2+} have been found to reduce or inhibit the majority of patch test reactions to simultaneously applied Ni^{2+} (18).

The aim of this study was to investigate the potential antagonistic effects of Ca^{2+} , Cu^{2+} , Fe^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} on the metal-ion-mediated lymphocyte proliferation in peripheral blood mononuclear cell (PBMC) cultures from contact-allergic subjects. The results from the *in vitro* experiments were further evaluated with Co^{2+} -sensitized guinea pigs.

Materials and Methods

Materials

Calcium chloride (CaCl_2), copper chloride ($\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$), iron chloride ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), magnesium chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), manganese chloride ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$), zinc chloride (ZnCl_2) (Aldrich Chemical Co., Milwaukee, WI, USA); Chelex-100 resin (Bio-Rad Laboratories, Richmond, CA, USA); $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (Merck KGaA, Darmstadt, Germany); gentamycin, L-glutamine, penicillin, RPMI 1640 medium and streptomycin (Gibco BRL, Life Technologies Ltd., Paisley, Scotland); phytohemagglutinin (PHA; Pharmacia Biotech., Uppsala, Sweden); propidium iodide (Sigma-Aldrich, Stockholm, Sweden); [methyl- ^3H]thymidine (Amersham International plc, Amersham Place, England); Freund's complete adjuvant (Difco, Detroit, MI, USA).

Subjects

6 female subjects (range of age: 25–53 years, mean: 35 years), with known contact allergy to cobalt or nickel, were recruited from the Karolinska Hospital. The patients had a positive patch test reaction to $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.5%) and $\text{NiSO}_4 \cdot 7\text{H}_2\text{O}$ (5.0%) in pet. (++: erythema, papules and infiltration or +++: erythema, papules, infiltration and vesicles), within 2 years before the start of this study. In addition, 6 female control subjects (range of age: 37–49 years, mean: 43 years), without a history of allergic contact dermatitis and with a nega-

tive patch test reaction to $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.5%) and $\text{NiSO}_4 \cdot 7\text{H}_2\text{O}$ (5.0%) in pet., participated in this study. All the subjects gave their informed consent to donate blood. The study was approved by the local ethics committee.

Lymphocyte proliferation assay

PBMCs were prepared from heparinized blood by Ficoll-Paque (Pharmacia-Biotech.) density gradient centrifugation. Cells from the interface were washed 3 times with RPMI 1640 medium and resuspended to 10^6 cells/ml in RPMI 1640 medium supplemented with heat-inactivated (56°C for 30 min) autologous serum (10%; v/v), gentamicin (25 $\mu\text{g}/\text{ml}$), penicillin (100 IU/ml), streptomycin (100 IU/ml) and L-glutamine (2 mM). PBMCs ($200 \mu\text{l}$; 2×10^5 cells/well) were cultured in triplicate in U-bottomed 96-microwell plates (Costar, Cambridge, MA, USA) at 37°C in an atmosphere of 5% CO_2 in air. After 30 min pre-incubation, 10 μl of freshly prepared filter-sterilised (pore: 0.20 μm ; Micro Filtration Systems, Dublin, CA, USA) solutions of Ca^{2+} , Cu^{2+} , Fe^{2+} , Mg^{2+} , Mn^{2+} or Zn^{2+} in saline (final concentration of 1–100 μM) or saline only was added to the cultures. The saline was pre-treated with Chelex-100 resin to remove possible transition metal ion contaminants. After 1 h at 37°C in an atmosphere of 5% CO_2 in air, 10 μl freshly prepared filter-sterilized Co^{2+} (10 μM) or Ni^{2+} (50 μM) in saline, PHA (10 $\mu\text{g}/\text{ml}$), or saline only was added. After a culture period of 96 h, the PBMC cultures were pulsed with 0.1 μCi of [methyl- ^3H]thymidine (specific activity: 25 Ci/mmol) in 10 μl RPMI 1640 medium. The cells were harvested after another 18 h on a Tomtec microplate harvester (Tomtec, Orange, CT, USA) using glass-fibre filters. The filters were dried and assayed in a Wallac 1205 betaplate scintillation counter (Wallac Oy, Turku, Finland).

Lymphocyte viability

In PBMC cultures from 5 control subjects the effect of Cu^{2+} (final concentration 1–10 μM) alone or in combination with Co^{2+} (10 μM) or Ni^{2+} (50 μM) on the PHA (10 $\mu\text{g}/\text{ml}$) induced lymphocyte proliferation was determined. After 72 h, the optimal culture period for PHA stimulated lymphocyte proliferation, the cells were pulsed with 0.1 μCi of [methyl- ^3H]thymidine and analyzed as described above. A decreased viability of the cells was defined as those metal ion concentrations which caused a statistically significant reduction of the PHA induced cellular [methyl- ^3H]thymidine uptake (2).

In addition, the viability of PBMCs cultured for

96 h with Cu^{2+} (1–10 μM) alone or in combination with Co^{2+} (10 μM) or Ni^{2+} (50 μM) was studied by FACS analysis using a FACSCalibur flow cytometer (Becton Dickinson). The cells were incubated for 15 min at 4°C with propidium iodide (final concentration of 5 $\mu\text{g}/\text{ml}$). The fluorescence was measured by acquiring 5000 to 10,000 events per sample. Viability of the cells was determined by analysing the percentage of cells which had not taken up propidium iodide.

Sensitization experiments in guinea pigs

The sensitization experiments were performed in outbred female albino Dunkin-Hartley guinea pigs obtained from AB Sahlins försöksdjursfarm, Malmö, Sweden. The animals were kept on a standard diet from EWOS AB, Södertälje, Sweden and their average weight was 300–350 g.

The experiments were carried out according to the modified Freund's complete adjuvant test (FCAT) (19). Closed challenge testing was performed according to previous experience (20). For induction the animals received intradermal injections of 0.1 ml of the test substance in FCA/water emulsion in the upper back on days 0, 6, and 10. The controls received FCA/water emulsion only. Challenge testing was performed on day 21 and rechallenge on day 63. The test material (15 μl of each dose) was applied on the shaved flanks for 24 h, using Finn chambers (aluminium chambers, 8 mm in diameter). The reactions were assessed at 48 and 72 h after application. The minimum criterium for a positive reaction was a confluent erythema.

The animals were randomized into 3 groups. Groups A ($n=15$) and B ($n=15$) were induced with $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 1% w/w, while group C ($n=15$) was a sham-treated control group. The induction concentration used was according to previous experience (21). The animals of groups A and C received a topical application of 110 mg of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 0.8% w/w in pet. on a total area of $4 \times 4 \text{ cm}^2$ of the left shaved flank 1 day prior to challenge testing. The same amount of pet. was applied on the animals right shaved flank. The application sites were occluded for 24 h. The animals in group B were pretreated in the same way with pet. on both flanks. The concentration of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ chosen was the maximum non-irritating as determined in preceding studies.

Challenge testing was performed in all groups with $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.1 and 0.3%, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 0.8% and with the vehicle pet. on both flanks at the same time using a blind randomized application system. The test concentrations of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ were chosen according to prior experience (21).

Pretreatment of groups B and C was performed 1 day prior to rechallenge. The animals received a topical application of ZnCl_2 0.1% w/w in pet. on their right shaved flank and pet. only on their left flank. The animals were occluded for 24 h. The animals in group A were pretreated in the same way with pet. on both flanks. All animals were tested with $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.1 and 0.3%, ZnCl_2 0.1%, and with the vehicle pet. on both flanks at the same time as described above. Before the treatment the maximum non-irritating concentration of ZnCl_2 was determined. One intradermal injection of FCA was given to each of 3 guinea pigs, which were tested with ZnCl_2 in concentrations 3.3–0.003% in pet. No irritation was seen to concentrations 0.1% and below.

Statistics

The statistical evaluation of the in vitro results was done with the Mann-Whitney U-test. The results of the animal experiments were analyzed with Fisher's exact test. A p -value <0.05 was considered statistically significant.

Results

Lymphocyte proliferation in PBMC cultures

The PBMC cultures from the contact-allergic subjects showed an increased cellular [methyl- ^3H]thymidine uptake by addition of Ni^{2+} or Co^{2+} (10–50 μM), in comparison with the cultures from the controls (Fig. 1). The strongest proliferative responses were detected with 50 μM Ni^{2+} and 10 μM Co^{2+} , which were used in all additional experiments. The polyclonal stimulant PHA (10 $\mu\text{g}/\text{ml}$),

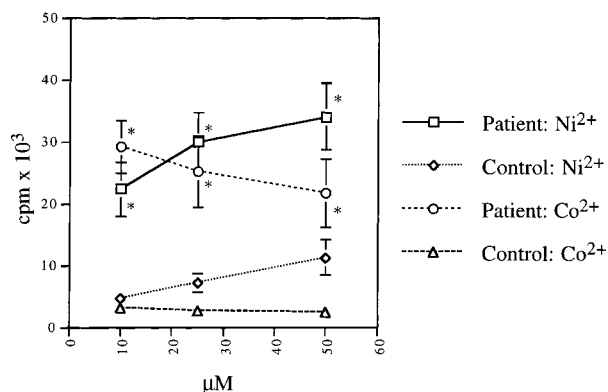


Fig. 1. Lymphocyte proliferation in PBMC cultures from patients contact-allergic to Co^{2+} and Ni^{2+} ($n=6$) and controls ($n=6$). PBMCs were cultured for 96 h in the presence of Co^{2+} or Ni^{2+} (10–50 μM). T-cell proliferation was measured by [methyl- ^3H]thymidine incorporation and expressed as mean $\text{cpm} \times 10^3 \pm \text{SE}$.

* Significantly different from the controls, $p < 0.01$.

used as a positive control, induced a lymphocyte proliferation of 115 ± 23 and 110 ± 23 cpm $\times 10^3$ (mean $\pm$ SE) in the cultures from the 6 patients and the 6 controls respectively. The background radioactivity of the vehicle (saline) treated cultures was 5.7 ± 1.3 cpm $\times 10^3$ for the allergic subjects and 4.3 ± 1.0 cpm $\times 10^3$ (mean $\pm$ SE) for the controls (Table 1).

The Ni²⁺- and Co²⁺-mediated lymphocyte proliferation in PBMC cultures from the allergic subjects was significantly reduced by addition of 5 μ M or more Cu²⁺ and 2.5 μ M or more Cu²⁺, respectively (Tables 1, 2). In addition, in the controls the uptake of [methyl-³H]thymidine was decreased by 10 μ M Cu²⁺ in comparison with Ni²⁺-treatment only, and by 5 μ M or more Cu²⁺ in comparison with Co²⁺-treatment only (Tables 1, 2). A difference between the patient and control groups was not measurable at a final Cu²⁺ concentration of 2.5, 5 or 10

Table 2. Effect of Cu²⁺, Zn²⁺, Ca²⁺, Fe²⁺, Mg²⁺ and Mn²⁺ on the Co²⁺-mediated lymphocyte proliferation in PBMC cultures from subjects contact-allergic to Co²⁺ and Ni²⁺

Treatment ^{a)}	Patients ^{b)} (n=6)	Controls ^{b)} (n=6)
vehicle (saline)	5.7 $\pm$ 1.3	4.3 $\pm$ 1.0
Co ²⁺ (10 μ M)	29.8 $\pm$ 4.5*	3.3 $\pm$ 0.6
+Cu ²⁺ (1 μ M)	21.1 $\pm$ 4.3*	2.9 $\pm$ 0.6
+Cu ²⁺ (2.5 μ M)	11.9 $\pm$ 3.0*§	1.9 $\pm$ 0.4
+Cu ²⁺ (5 μ M)	5.0 $\pm$ 2.2§§	1.3 $\pm$ 0.2§§
+Cu ²⁺ (10 μ M)	1.9 $\pm$ 0.7§§	1.0 $\pm$ 0.2§§
+Zn ²⁺ (10 μ M)	26.9 $\pm$ 4.2*	7.3 $\pm$ 1.5§
+Zn ²⁺ (25 μ M)	30.8 $\pm$ 4.5*	10.9 $\pm$ 1.4§§
+Zn ²⁺ (50 μ M)	41.8 $\pm$ 7.3*	17.4 $\pm$ 2.0§§
+Zn ²⁺ (100 μ M)	73.6 $\pm$ 19.9§	60.0 $\pm$ 7.9§§
+Ca ²⁺ (10 μ M)	27.9 $\pm$ 5.7*	3.7 $\pm$ 0.6
+Ca ²⁺ (25 μ M)	24.9 $\pm$ 4.1*	3.7 $\pm$ 0.4
+Ca ²⁺ (50 μ M)	25.6 $\pm$ 4.2*	3.9 $\pm$ 0.9
+Ca ²⁺ (100 μ M)	26.0 $\pm$ 4.1*	4.1 $\pm$ 0.8
+Fe ²⁺ (10 μ M)	28.9 $\pm$ 5.9*	3.0 $\pm$ 0.3
+Fe ²⁺ (25 μ M)	25.0 $\pm$ 5.8*	3.3 $\pm$ 0.5
+Fe ²⁺ (50 μ M)	25.0 $\pm$ 5.2*	2.6 $\pm$ 0.4
+Fe ²⁺ (100 μ M)	24.4 $\pm$ 5.1*	2.8 $\pm$ 0.4
+Mg ²⁺ (10 μ M)	27.2 $\pm$ 6.6*	4.3 $\pm$ 0.7
+Mg ²⁺ (25 μ M)	30.0 $\pm$ 8.0*	4.7 $\pm$ 0.8
+Mg ²⁺ (50 μ M)	26.2 $\pm$ 5.1*	5.0 $\pm$ 1.2
+Mg ²⁺ (100 μ M)	26.9 $\pm$ 4.9*	3.9 $\pm$ 0.9
+Mn ²⁺ (1 μ M)	26.4 $\pm$ 6.3*	3.6 $\pm$ 0.7
+Mn ²⁺ (2.5 μ M)	25.9 $\pm$ 4.9*	3.8 $\pm$ 0.7
+Mn ²⁺ (5 μ M)	20.9 $\pm$ 4.2*	3.9 $\pm$ 1.0
+Mn ²⁺ (10 μ M)	17.7 $\pm$ 4.5*	3.0 $\pm$ 0.6

a) PBMCs were cultured for 96 h in the presence of Co²⁺ (10 μ M) + different amounts of Cu²⁺, Mn²⁺ (1–10 μ M) or Ca²⁺, Fe²⁺, Mg²⁺, Zn²⁺ (10–100 μ M).

b) T-cell proliferation was measured by [methyl-³H]-thymidine incorporation and expressed as mean cpm $\times 10^3 \pm$ SE.

* Significantly different from the controls, $p < 0.01$.

§ Significantly different from the Co²⁺ treatment only, $p < 0.05$.

§§ Significantly different from the Co²⁺ treatment only, $p < 0.01$.

Table 1. Effect of Cu²⁺, Zn²⁺, Ca²⁺, Fe²⁺, Mg²⁺ and Mn²⁺ on the Ni²⁺-mediated lymphocyte proliferation in PBMC cultures from subjects contact-allergic to Co²⁺ and Ni²⁺

Treatment ^{a)}	Patients ^{b)} (n=6)	Controls ^{b)} (n=6)
vehicle (saline)	5.7 $\pm$ 1.3	4.3 $\pm$ 1.0
Ni ²⁺ (50 μ M)	34.0 $\pm$ 5.4**	11.3 $\pm$ 3.0
+Cu ²⁺ (1 μ M)	31.0 $\pm$ 4.8**	12.3 $\pm$ 2.7
+Cu ²⁺ (2.5 μ M)	20.8 $\pm$ 4.6	11.4 $\pm$ 3.4
+Cu ²⁺ (5 μ M)	8.4 $\pm$ 1.8§§	8.0 $\pm$ 3.8
+Cu ²⁺ (10 μ M)	3.5 $\pm$ 1.1§§	3.7 $\pm$ 0.8§
+Zn ²⁺ (10 μ M)	33.7 $\pm$ 6.1*	13.7 $\pm$ 4.5
+Zn ²⁺ (25 μ M)	39.4 $\pm$ 6.9*	16.9 $\pm$ 3.5
+Zn ²⁺ (50 μ M)	44.7 $\pm$ 8.0	24.1 $\pm$ 5.5
+Zn ²⁺ (100 μ M)	67.6 $\pm$ 12.9§	53.7 $\pm$ 12.5§§
+Ca ²⁺ (10 μ M)	32.7 $\pm$ 6.3*	11.9 $\pm$ 5.0
+Ca ²⁺ (25 μ M)	33.0 $\pm$ 6.6*	12.2 $\pm$ 5.0
+Ca ²⁺ (50 μ M)	33.4 $\pm$ 5.7*	13.0 $\pm$ 4.7
+Ca ²⁺ (100 μ M)	33.9 $\pm$ 7.2**	12.5 $\pm$ 2.8
+Fe ²⁺ (10 μ M)	31.0 $\pm$ 5.1*	12.4 $\pm$ 3.3
+Fe ²⁺ (25 μ M)	30.0 $\pm$ 5.6*	12.3 $\pm$ 2.9
+Fe ²⁺ (50 μ M)	29.7 $\pm$ 5.7*	12.5 $\pm$ 3.9
+Fe ²⁺ (100 μ M)	29.7 $\pm$ 5.3*	12.4 $\pm$ 2.3
+Mg ²⁺ (10 μ M)	31.2 $\pm$ 5.9*	11.3 $\pm$ 3.7
+Mg ²⁺ (25 μ M)	32.3 $\pm$ 5.1*	12.6 $\pm$ 4.1
+Mg ²⁺ (50 μ M)	32.1 $\pm$ 4.5*	12.7 $\pm$ 3.6
+Mg ²⁺ (100 μ M)	30.6 $\pm$ 4.2*	12.5 $\pm$ 4.1
+Mn ²⁺ (1 μ M)	30.6 $\pm$ 7.0**	11.2 $\pm$ 2.5
+Mn ²⁺ (2.5 μ M)	31.2 $\pm$ 5.5*	12.5 $\pm$ 2.7
+Mn ²⁺ (5 μ M)	29.9 $\pm$ 5.7**	11.5 $\pm$ 2.1
+Mn ²⁺ (10 μ M)	26.1 $\pm$ 5.0*	9.3 $\pm$ 1.7

a) PBMCs were cultured for 96 h in the presence of Ni²⁺ (50 μ M) + different amounts of Cu²⁺, Mn²⁺ (1–10 μ M) or Ca²⁺, Fe²⁺, Mg²⁺, Zn²⁺ (10–100 μ M).

b) T-cell proliferation was measured by [methyl-³H]-thymidine incorporation and expressed as mean cpm $\times 10^3 \pm$ SE.

* Significantly different from the controls, $p < 0.05$.

** Significantly different from the controls, $p < 0.01$.

§ Significantly different from the Ni²⁺ treatment only, $p < 0.05$.

§§ Significantly different from the Ni²⁺ treatment only, $p < 0.01$.

μ M for Ni²⁺ (Table 1) and a Cu²⁺ concentration of 5 or 10 μ M for Co²⁺ (Table 2). The cellular [methyl-³H]thymidine incorporation from the allergic individuals and controls was not increased or decreased by Cu²⁺ only (without Ni²⁺ or Co²⁺) in comparison with the vehicle treated cultures (results not presented). The PHA (10 μ g/ml) induced [methyl-³H]thymidine incorporation by control PBMCs was not increased or decreased by Cu²⁺ (1–10 μ M), Ni²⁺ (50 μ M) or Co²⁺ (10 μ M) separately (Fig. 2a). However, the cell proliferation was significantly reduced by applied combinations of Ni²⁺ (50 μ M) with Cu²⁺ (2.5–10 μ M) or Co²⁺ (10 μ M) with Cu²⁺ (1–10 μ M) (Fig. 2a). In addition, FACS analysis of propidium iodide (5 μ g/ml) treated PBMCs showed a significant reduced cell viability by applied combinations of Ni²⁺ (50 μ M) or Co²⁺ (10 μ M) with Cu²⁺ (10 μ M), while Cu²⁺ (1–10 μ M), Ni²⁺ (50 μ M) or Co²⁺ (10 μ M) separately was not toxic to the cells (Fig. 2b).

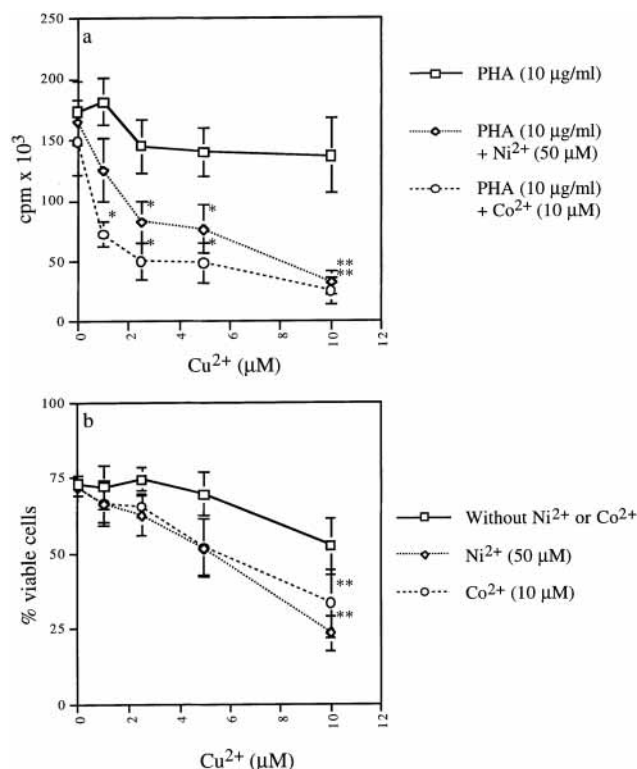


Fig. 2. The effect of Cu^{2+} (1–10 μM) alone or in combination with Co^{2+} (10 μM) or Ni^{2+} (50 μM) on lymphocytes from controls ($n=5$). (a) Lymphocyte proliferation of PBMC cultures incubated for 72 h in the presence of PHA (10 $\mu\text{g/ml}$). T-cell proliferation was measured by [methyl- ^3H]thymidine incorporation and expressed as mean $\text{cpm} \times 10^3 \pm \text{SE}$. (b) FACS analysis of PBMCs cultured for 96 h. The cells were incubated for 15 min at 4°C with propidium iodide (final concentration of 5 $\mu\text{g/ml}$). Viability of the cells was determined by analyzing the percentage of cells which had not taken up propidium iodide.

* Significantly different from the treatment without Cu^{2+} , Co^{2+} or Ni^{2+} , $p < 0.05$.

** Significantly different from the treatment without Cu^{2+} , Co^{2+} or Ni^{2+} , $p < 0.01$.

Treatment of cultures from the allergic individuals and the control subjects with Zn^{2+} (100 μM), in combination with Ni^{2+} (50 μM) or Co^{2+} (10 μM), resulted in significant enhancement of the [methyl- ^3H]thymidine uptake, in comparison with Ni^{2+} - or Co^{2+} -treatment only (Tables 1, 2). As a consequence, a difference in lymphocyte proliferation between the patient and control group was no longer apparent (Tables 1, 2). Also Zn^{2+} only (without Ni^{2+} or Co^{2+}) increased the [methyl- ^3H]thymidine uptake in comparison with the vehicle treated cultures, $17.1 \pm 4.6 \text{ cpm} \times 10^3$ (mean $\pm \text{SE}$; $p < 0.05$) with 50 μM Zn^{2+} and $45.4 \pm 11.2 \text{ cpm} \times 10^3$ ($p < 0.01$) with 100 μM Zn^{2+} for the patients, and $16.5 \pm 4.2 \text{ cpm} \times 10^3$ ($p < 0.05$) with 50 μM Zn^{2+} and $48.6 \pm 11.3 \text{ cpm} \times 10^3$ ($p < 0.01$) with 100 μM Zn^{2+} for the control subjects. In the cultures from the patients, the lympho-

cyte proliferation induced by the combination of Ni^{2+} or Co^{2+} hapten with Zn^{2+} (50 μM) was statistically higher than the one induced by Zn^{2+} (50 μM) only ($p < 0.05$).

Ca^{2+} , Fe^{2+} , Mg^{2+} and Mn^{2+} did not influence the Ni^{2+} - and Co^{2+} -mediated lymphocyte proliferation in the cultures from contact-allergic individuals and controls (Tables 1, 2). Addition of these metal ions only (without Ni^{2+} and Co^{2+}) did not increase or decrease the [methyl- ^3H]thymidine uptake in comparison with the vehicle-treated cultures (results not presented).

Effect of Cu^{2+} and Zn^{2+} on the response to Co^{2+} in sensitized guinea pigs

The animals induced with $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ became clearly sensitized. A significant response was found at challenge testing with 0.3 and 0.1% (Tables 3, 4). No irritation from $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ was observed in the controls at 1st challenge, but some reactions were found at rechallenge which could have been due to sensitization at the 1st challenge testing or increased irritation in the skin (Table 4, group C). No difference in reactivity to $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ was observed between the flank pretreated with $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.8%) and the vehicle pretreated flank (Table 3, group A). In addition, no difference was seen in reactivity between the animals pretreated with $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (group A) and the animals pretreated with the vehicle only (group B). Pretreatment with $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ did not affect irritation since no reactions were found on either flank of the controls (group C).

Pretreatment with ZnCl_2 did not affect the reactivity to $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (Table 4). No difference was observed between either the ZnCl_2 -pretreated and vehicle-pretreated flanks (group B), or the ZnCl_2 -pretreated and vehicle-pretreated animal groups (groups B and A). The animals in group A showed a significant response after challenge testing with ZnCl_2 on one flank, while no significance was observed on the other flank.

Discussion

In this study the potential antagonistic effects of a series of 6 physiologically relevant divalent metal ions on contact allergy to Ni^{2+} and Co^{2+} were investigated. Like Ni^{2+} and Co^{2+} , Cu^{2+} favours forming complexes with histidine- and cysteine-containing residues of proteins (12). Cu^{2+} (5–10 μM) markedly reduced the Co^{2+} (10 μM) and Ni^{2+} (50 μM) induced [methyl- ^3H]thymidine incorporation in the cultures from the contact-allergic subjects (Tables 1, 2). However, this potential antagon-

Table 3. Effect of pretreatment with Cu^{2+} at challenge testing in Co^{2+} -sensitized guinea pigs

	Challenge material			
	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.3% ^{a)}	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.1% ^{a)}	$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 0.8% ^{a)}	pet.
Exposed groups ^{b)}				
Group A ($n=15$) ^{c)}				
pretreated with $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.8% ^{a)})	12**	4*	0	0
pretreated with pet.	11**	4*	0	0
Group B ($n=15$) ^{d)}				
pretreated with pet.	11**	4*	1	1
pretreated with pet.	12**	4*	1	0
Controls ^{b)}				
Group C ($n=15$) ^{e)}				
pretreated with $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.8% ^{a)})	0	0	0	0
pretreated with pet.	0	0	0	0

a) % w/w in pet.

b) The number of animals with positive reactions (++) and (+++) at 72 h after application of the challenge material is presented. (The reactivity at 48 h after application did not differ significantly from these data.)

c) Prior to the challenge test, Co^{2+} -sensitized animals received a 24 h closed epidermal application of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.8% w/w) on one side of the shaven flanks and pet. only on the other side.d) Prior to the challenge test, Co^{2+} -sensitized animals received a 24 h closed epidermal application of pet. on both sides of the shaven flanks.e) Prior to the challenge test, control animals received a 24 h closed epidermal application of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.8% w/w) on one side of the shaven flanks and pet. only on the other side.* Significantly different from the controls $p < 0.05$.** Significantly different from the controls $p < 0.001$.Table 4. Effect of pretreatment with Zn^{2+} at re-challenge testing with Co^{2+} sensitized guinea pigs

	Challenge material			
	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.3% ^{a)}	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.1% ^{a)}	$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1% ^{a)}	pet.
Exposed groups ^{b)}				
Group A ($n=15$) ^{d)}				
pretreated with pet.	11**	9*	5*	3
pretreated with pet.	13**	7*	3	2
Group B ($n=15$) ^{c)}				
pretreated with pet.	12* *	8*	2	2
pretreated with ZnCl_2 (0.1% ^{a)})	10**	6*	0	2
Controls ^{b)}				
Group C ($n=15$) ^{e)}				
pretreated with pet.	1	2	0	1
pretreated with ZnCl_2 (0.1% ^{a)})	0	1	0	0

a) % w/w in pet.

b) The number of animals with positive reactions (++) and (+++) at 72 h after application of the challenge material is presented. (The reactivity at 48 h after application did not differ significantly from these data.)

c) Prior to the challenge test, Co^{2+} -sensitized animals received a 24 h closed epidermal application of ZnCl_2 (0.1% w/w) on one side of the shaven flanks and pet. only on the other side.d) Prior to the challenge test, Co^{2+} -sensitized animals received a 24 h closed epidermal application of pet. on both sides of the shaven flanks.e) Prior to the challenge test, control animals received a 24 h closed epidermal application of ZnCl_2 (0.1% w/w) on one side of the shaven flanks and pet. only on the other side.* Significantly different from the controls $p < 0.05$.** Significantly different from the controls $p < 0.001$.

istic effect of Cu^{2+} could not be reproduced in Co^{2+} sensitized guinea pigs tested according to the Freund's complete adjuvant test (Table 3). As indicated by the markedly reduced PHA (10 $\mu\text{g}/\text{ml}$) mediated lymphocyte proliferation in vitro and by

results obtained by FACS analysis of propidium iodide treated PBMCs, the viability of the cells was significantly affected by added combinations of Cu^{2+} with Co^{2+} or Ni^{2+} (Fig. 2a, 2b). Accordingly, we suspect that the inhibiting effect of Cu^{2+}

on the Ni^{2+} - and Co^{2+} -induced lymphocyte proliferation in vitro was related to decreased lymphocyte viability. Besides, some Ni^{2+} -reactive T cell clones have been found to cross-react with Cu^{2+} , while other clones did not (2, 5). Probably due to the limited number of allergic individuals tested in our study, we did not observe a case of Cu^{2+} cross-reactivity.

Histidine is by far the most frequently observed binding ligand in zinc enzymes. But glutamate, aspartate and cysteine are also found in catalytic Zn^{2+} sites (22). Co^{2+} derivatives of Zn^{2+} -utilizing enzymes often exhibit considerable catalytic activities as demonstrated for, e.g., carboxypeptidase A and astacin (12–14). Although Ni^{2+} -substituted astacin is almost inactive (14), the peptidase and esterase activities of the Ni^{2+} -derivative of carboxypeptidase A was comparable to that of the native Zn^{2+} -containing enzyme (13). Despite the above-illustrated apparent resemblances in binding properties, Zn^{2+} did not reduce the challenge test response to Co^{2+} (Table 4), and even significantly enhanced the Co^{2+} - and Ni^{2+} -mediated [methyl- ^3H]thymidine incorporation in the cultures from the contact-allergic individuals (Tables 1, 2). In addition, Zn^{2+} (50–100 μM) only (without Co^{2+} or Ni^{2+}) also stimulated the lymphocyte activity in vitro. Although Zn^{2+} is not known as a contact allergen, possible cross-reactivity of Zn^{2+} with Co^{2+} and Ni^{2+} was excluded since Zn^{2+} -induced [methyl- ^3H]thymidine incorporation was also observed in control cultures. Activation of human PBMCs by Zn^{2+} has been found by others and is probably triggered by interaction of Zn^{2+} with monocyte, leading to stimulated secretion of monokines (e.g., IL-6 and IL-1 β) (23). The positive challenge response to Zn^{2+} in one group on one side of Co^{2+} -sensitized animals (Table 4, group A) might thus also be related to Zn^{2+} -induced epidermal monokine release. Clearly, the maximum non-irritating dose of Zn^{2+} was slightly exceeded by this particular treatment. It has long been recognized that Zn^{2+} has an important role in the functioning of the immune system (24), but the significance of the observed T cell stimulation is unclear.

Ca^{2+} , Fe^{2+} , Mg^{2+} and Mn^{2+} did not decrease or increase the Co^{2+} - and Ni^{2+} -mediated [methyl- ^3H]thymidine incorporation in vitro (Tables 1, 2). With respect to supposed antagonism to Ni^{2+} contact allergy from, e.g., Mg^{2+} (18), conflicting results have been presented since only MgSO_4 but not MgCl_2 appeared to reduce or inhibit patch test reactions to Ni^{2+} (25).

In conclusion, our combined results obtained with the human in vitro antigen-induced lymphocyte proliferation and the Freund's complete adju-

vant test in guinea pigs indicate that Ca^{2+} , Cu^{2+} , Fe^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} are not able to antagonise the formation of Ni^{2+} and Co^{2+} antigens.

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