Acta Derm Venereol (Stockh) 1998; 78: 95–98

Free Radicals as Potential Mediators of Metal Allergy: Effect of Ascorbic Acid on Lymphocyte Proliferation and IFN-γ Production in Contact Allergy to Ni^{2+} and Co^{2+}

LEON T. VAN DEN BROEKE[1,2], ASTRID GRÄSLUND[3], PER H. LARSSON[4], J. LARS G. NILSSON[1], JAN E. WAHLBERG[1,5], ANNIKA SCHEYNIUS[2] and ANN-THERESE KARLBERG[1]

[1]Department of Occupational Health, Dermatology Division, National Institute for Working Life, [2]Department of Laboratory Medicine, Division of Clinical Immunology, Karolinska Institute and Hospital, [3]Department of Biophysics, Stockholm University, [4]Department of Occupational Health, Division for Respiratory Allergy, National Institute for Working Life, and [5]Department of Environmental and Occupational Dermatology, Karolinska Hospital, Stockholm, Sweden

A possible free radical mechanism in metal allergy was investigated in peripheral blood mononuclear cell (PBMC) cultures from 6 subjects, contact allergic to Ni^{2+} and Co^{2+}, and 6 control individuals. Ni^{2+} and Co^{2+}-mediated free radical generation was studied with electron spin resonance spectroscopy. The immune response was characterized by cellular [methyl-^{3}H]thymidine uptake and interferon-γ (IFN-γ) production. Ni^{2+} and Co^{2+} (10–50 μM) significantly increased lymphocyte proliferation and IFN-γ production in PBMC cultures from contact allergic subjects in comparison with cultures from controls. Inhibition of Co^{2+}-mediated free radical generation by ascorbic acid did not influence cellular [methyl-^{3}H]thymidine uptake and IFN-γ production. Detectable amounts of free radicals were not obtained with Ni^{2+}. We therefore conclude that it is unlikely that free radicals are involved in contact allergy to Ni^{2+} and Co^{2+}. *Key words: metal-ion; ESR spectroscopy; peripheral blood mononuclear cells; human.*

(Accepted August 7, 1997.)

Acta Derm Venereol (Stockh) 1998; 78: 95–98.

L. T. Van den Broeke, Department of Occupational Health, Dermatology Division, National Institute for Working Life, S-171 84 Solna, Sweden.

Although Ni^{2+} and Co^{2+} are two of the most frequent causes of allergic contact dermatitis (ACD) (1), the pathogenesis is still essentially unknown. As a result, apart from symptomatic treatment with e.g. corticosteroids, specific methods of treating ACD to Ni^{2+} and Co^{2+} are at present not available. Analysis of the early events leading to ACD to Ni^{2+} and Co^{2+} may lead to new ways for prevention and treatment.

Studies with Co^{2+}-sensitized guinea-pigs (2) and human Ni^{2+}-reactive T-lymphocyte clones (3) showed that Ni^{2+} and Co^{2+} do not cross-react. Simultaneous reactivity to Ni^{2+} and Co^{2+}, which is often seen in contact allergic individuals, is therefore most likely the result of separate sensitizations. In a previous paper, we have described that the two metal-ions, when chelated with proper ligands, are able to generate significant amounts of free radicals under biologically relevant conditions (4). Comparable results have been obtained by others (5, 6). Ni^{2+}- and Co^{2+}-mediated free radical production has also been observed *in vivo*, in circulating blood in rats (7). However, we found that the free radical-generating properties of Ni^{2+} and Co^{2+} were distinctly different (4). For example, at physiological pH glutathione and histidine significantly enhanced free radical generation of Co^{2+} from H_2O_2, whereas under identical conditions the reactivity of Ni^{2+} toward H_2O_2 was not changed. We considered this difference in free radical-generating properties between Ni^{2+} and Co^{2+} to be of interest with respect to the absence of cross-reactivity between the two metal-ions in experimental ACD.

In biological tissues, the free radicals derived from metal-ion-mediated redox reactions are likely to react further with biomolecules close to their generation site. We hypothesize that those secondary reactions lead to the formation of antigenic structural modifications in biomolecules and as such ultimately cause ACD to Ni^{2+} and Co^{2+}. This hypothesis differs from the dominating theory of the initial step of antigen formation in ACD to metal-ions, namely the formation of specific coordination complexes with electron-rich ligands (8). However, a possible free radical mechanism of antigen formation has been suggested for other haptens, such as hydroperoxides and urushiols (9, 10). A free radical-mediated mechanism in ACD to Ni^{2+} and Co^{2+} has so far not been investigated.

The aim of this paper was to study the free radical-scavenging effect of ascorbic acid on metal-ion-mediated lymphocyte proliferation and interferon-γ (IFN-γ) production in peripheral blood mononuclear cell (PBMC) cultures from contact allergic patients and control subjects.

MATERIALS AND METHODS

Materials

L-Ascorbic acid sodium salt, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (Sigma Chemical Co., St. Louis, MO); Chelex-100 resin (Bio-Rad Laboratories, Richmond, CA); $CoCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$ (Merck KGaA, Darmstadt, Germany); gentamycin, L-glutamine, penicillin, RPMI 1640 medium, streptomycin (Gibco BRL, Life Technologies Ltd., Paisley, Scotland); phytohemagglutinin (PHA) (Pharmacia Biotech., Uppsala, Sweden) and [methyl-^{3}H]thymidine (Amersham International plc, Amersham Place, England) were used in this study. The patch-test materials were obtained from Chemotechnique Diagnostics (Malmö, Sweden) and the subjects were tested using Finn chambers according to a standard procedure (11).

Subjects

Six female subjects (age range: 24–52 years, mean: 33 years), with confirmed ACD to materials containing cobalt or nickel and with positive patch-test reactions (++: erythema, papules and infiltration or +++: erythema, papules, infiltration and vesicles) to $CoCl_2 \cdot 6H_2O$ (0.5%) and to $NiSO_4 \cdot 7H_2O$ (5.0%) in petrolatum, were recruited from the Karolinska Hospital. The patients had had a positive patch-test within 1 year before the start of the study. In addition, 6 female control subjects (age range: 37–49 years, mean: 45 years), without a history of ACD participated in the study. When subjected to patch-testing they all had a negative patch-test reaction to $CoCl_2 \cdot 6H_2O$, but 3 of them showed a weak positive patch-test reaction (+: erythema

 ISSN 0001-5555

and oedema) to $NiSO_4 \cdot 7H_2O$. The *in vitro* Ni^{2+} and Co^{2+}-induced lymphocyte proliferation, IFN-γ production and free radical generation were similar for all 6 controls subjects. The + patch-test reaction to $NiSO_4 \cdot 7H_2O$ was regarded as not clinically relevant (11–13). Thus, the controls were considered as one population. All the subjects gave their informed consent to donate blood. The study was approved by the local ethics committee.

Preparation of PBMC cultures

Ficoll-Paque (Pharmacia-Biotech., Uppsala, Sweden) density gradient centrifugation was used to prepare PBMCs from heparinized blood. Cells from the interface were washed three times with RPMI 1640 medium and resuspended to 10^6 cells/ml in RPMI 1640 medium, supplemented with heat-inactivated autologous serum (10%; v/v), gentamycin (25 μg/ml), penicillin (100 IU/ml), streptomycin (100 IU/ml) and L-glutamine (2 mM). The PBMCs (200 μl; 2×10^5 cells/well) were cultured in U-bottomed 96-microwell plates (Costar, Cambridge, MA) at 37°C in an atmosphere of 5% CO_2 in air. After pre-incubation for 30 min, 10 μl of filter-sterilized ascorbic acid (final concentration 20–100 μM) in phosphate-buffered saline (PBS; pH = 7.4, 10 mM) or PBS only was added. Next, 10 μl filter-sterilized Co^{2+} or Ni^{2+} (final concentration 10–50 μM) in saline, phytohemagglutinin (PHA; final concentration 10 μg/ml) or saline only was added. The saline and PBS were pretreated with Chelex-100 resin to remove possible transition metal-ion contaminants.

Lymphocyte proliferation assay

Initial experiments indicated that optimal lymphocyte proliferation was obtained after a culture period of 4.5 days. Thus, after 96 h the PBMC cultures were pulsed with 0.1 μCi of [methyl-^{3}H]thymidine (specific activity: 25 Ci/mmol) in 10 μl RPMI 1640 medium. The cells were harvested after another 18 h on a Skatron multiple-cell collector, using glass-fibre filters. The filters were dried and assayed in a Packard liquid scintillation counter. The background radioactivity of the vehicle- (PBS + saline) treated cultures was 3,424 ± 760 CPM (mean ± SE) for the allergic subjects and 1,947 ± 477 CPM for the controls. The lymphocyte proliferation assay was performed in triplicate.

IFN-γ assay

The highest IFN-γ levels were obtained after a culture period of 4 days, as determined by preceding experiments. Consequently, after a culture period of 96 h supernatants of the PBMC cultures were taken by centrifugation (10 min; 400 G) and stored at −80°C until used. The IFN-γ levels were analyzed in duplicate with an ELISA procedure according to a standard protocol from Mabtech AB (Stockholm, Sweden). The sensitivity of the assay was enhanced up to 4 pg/ml by amplification with N-biotinyl-4-hydroxybenzohydrazone (14). The colour reaction was measured in a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA) and evaluated with the Softmax Pro Software (Molecular Devices).

Electron spin resonance (ESR) measurements

According to a recently described procedure (4), free radical generation in PBMC cultures was studied by spin trapping with DMPO (final concentration 200 mM) in a Varian E9 ESR spectrometer. After 3 h incubation (unless differently indicated) duplicate samples were analyzed.

Statistics

The statistical evaluation of the results was performed with the Mann-Whitney U-test.

RESULTS

Lymphocyte proliferation and IFN-γ production

Addition of Ni^{2+} and Co^{2+} to PBMC cultures from the contact allergic subjects significantly increased the cellular [methyl-^{3}H]thymidine uptake and the production of IFN-γ, in comparison with the cultures from controls (Fig. 1a,b). The polyclonal stimulant PHA (10 μg/ml) induced a lymphocyte proliferation of 94,834 ± 18,299 CPM (mean ± SE) and an IFN-γ production of 2,814 ± 586 pg/ml in the cultures from the allergic subjects. With the control subjects these amounted 70,091 ± 6,103 CPM and 1,373 ± 201 pg/ml, respectively. The PHA-induced cellular uptake of [methyl-^{3}H]thymidine and the production of IFN-γ were significantly different from the vehicle-treated cultures ($p < 0.005$).

ESR measurements

Free radical production in the PBMC cultures increased linearly with increasing Co^{2+} dose. No difference in intensity of the ESR signal was observed between cultures from patients and controls (Fig. 2). Pretreatment of the PBMC cultures with ascorbic acid resulted in the absence of a detectable spin-trap

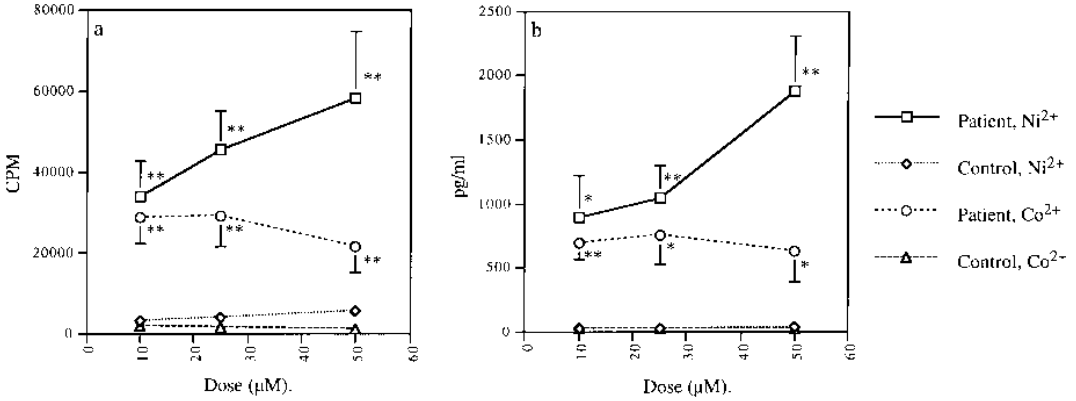

Fig. 1. Lymphocyte proliferation (*a*) and IFN-γ production (*b*) in PBMC cultures from patients contact allergic to Co^{2+} and Ni^{2+} ($n = 6$) and controls ($n = 6$). PBMCs were cultured in the presence of Co^{2+} or Ni^{2+} (10–50 μM). After 4.5 days the T-cell proliferation was measured by [methyl-^{3}H]thymidine incorporation and expressed as mean CPM ± SE. IFN-γ levels in the supernatants were measured after 4 days with an ELISA method and expressed as mean pg/ml ± SE. *: significantly different from the controls, $p < 0.05$; **: significantly different from the controls, $p < 0.01$.

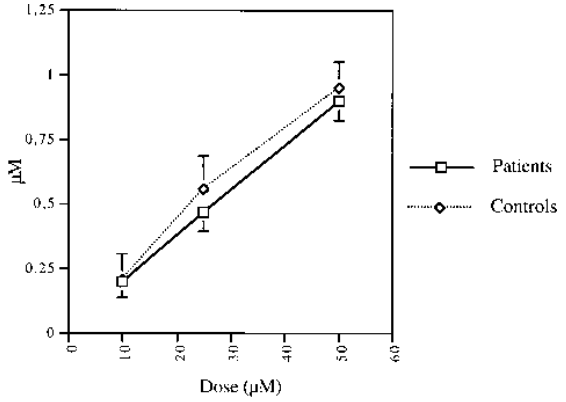

Fig. 2. Free radical production in PBMC cultures from patients contact allergic to Co^{2+} and Ni^{2+} ($n=6$) and controls ($n=6$). PBMCs were cultured for 3 h in the presence of Co^{2+} (10–50 µM) and DMPO (200 mM). Free radicals were measured with ESR spectroscopy and expressed as mean µM ± SE.

adduct of DMPO, indicating an efficient inhibition of Co^{2+}-generated free radicals. Ni^{2+}, even at a concentration of 100 µM, did not generate detectable amounts of free radicals under the applied culture conditions (data not presented).

Effect of ascorbic acid on Ni^{2+}- and Co^{2+}-induced lymphocyte proliferation and IFN-γ production

Pretreatment of the PBMC cultures from allergic subjects and controls with ascorbic acid did not influence Ni^{2+}- and Co^{2+}-induced [methyl-^{3}H]-thymidine incorporation and IFN-γ production. Consequently, cultures from allergic subjects incorporated [methyl-^{3}H]thymidine at significantly higher rates and produced IFN-γ in significantly higher amounts on application of metal-ion + ascorbic acid, in comparison with the cultures from controls (Table I).

DISCUSSION

In this paper we showed that Ni^{2+} and Co^{2+} (10–50 µM) increased the cellular [methyl-^{3}H]thymidine uptake and IFN-γ production in PBMC cultures from individuals with clinically relevant contact allergy to both metal-ions. However, inhibition of metal-ion-mediated free radical generation by ascorbic acid did not affect the immune response. A free radical involvement in ACD to Ni^{2+} and Co^{2+} is therefore unlikely.

The metal-ion-induced lymphocyte proliferation in PBMC cultures of allergic subjects (Fig. 1a) is in accordance with observations by others (12, 15, 16). Although Ni^{2+}-induced production of high levels of IFN-γ has been found with Ni^{2+}-specific T-cell clones (16), studies with PBMC cultures from Ni^{2+}-allergic individuals have been unsuccessful (17). This discrepancy with our results (Fig. 1b) is possibly related to differences in experimental protocols. Karttunen et al. (17) collected supernatants after culture periods of 5 and 7 days, whereas we found a rapid decrease in IFN-γ levels after an incubation period of 4 days, with no apparent enhanced concentration after 7 days of incubation (data not presented).

In the present study, 3 of the 6 control subjects showed a weak patch-test response (+: erythema and oedema) to $NiSO_4 \cdot 7H_2O$ (5.0%) in petrolatum. In a normal population, without clinical signs of ACD to nickel, positive patch-test responses to $NiSO_4 \cdot 7H_2O$ have been observed before (11). This phenomenon is typical of $NiSO_4 \cdot 7H_2O$ and much less frequently apparent with other metal-ion haptens like

Table I. *Effect of ascorbic acid on lymphocyte proliferation and interferon-γ production in peripheral blood mononuclear cell cultures from contact allergic subjects and controls*

Co^{2+}[a]	Ascorbic acid[a]	Lymphocyte proliferation		Interferon-γ production	
		Patients CPM ± SE[b]	Controls CPM ± SE[b]	Patients pg/ml ± SE[c]	Controls pg/ml ± SE[c]
0 µM	100 µM	3,390 ± 240 ($n=6$)	2,376 ± 189 ($n=6$)	31 ± 7 ($n=6$)	34 ± 12 ($n=6$)
10	20	26,122 ± 7,223* ($n=5$)	2,101 ± 292 ($n=5$)	584 ± 99** ($n=5$)	18 ± 9 ($n=5$)
	100	33,403 ± 8,053** ($n=6$)	1,833 ± 345 ($n=6$)	688 ± 226* ($n=6$)	22 ± 9 ($n=6$)
25	50	28,409 ± 9,047* ($n=5$)	1,401 ± 188 ($n=5$)	584 ± 162* ($n=5$)	16 ± 7 ($n=5$)
	100	26,120 ± 5,699** ($n=6$)	1,319 ± 351 ($n=6$)	705 ± 274* ($n=6$)	26 ± 15 ($n=6$)
50	100	19,701 ± 6,074* ($n=6$)	1,035 ± 241 ($n=6$)	624 ± 264* ($n=6$)	18 ± 9 ($n=6$)
Ni^{2+}[a]	Ascorbic acid[a]	Patients CPM ± SE[b]	Controls CPM ± SE[b]	Patients pg/ml ± SE[c]	Controls pg/ml ± SE[c]
10 µM	20 µM	25,602 ± 6,792* ($n=5$)	2,753 ± 673 ($n=5$)	703 ± 251* ($n=5$)	17 ± 7 ($n=5$)
	100	29,644 ± 9,932* ($n=6$)	2,701 ± 695 ($n=6$)	598 ± 266* ($n=6$)	23 ± 8 ($n=6$)
25	50	35,052 ± 8,401* ($n=5$)	3,743 ± 635 ($n=5$)	935 ± 171** ($n=5$)	32 ± 16 ($n=5$)
	100	33,521 ± 6,247** ($n=6$)	3,904 ± 1,451 ($n=6$)	902 ± 241** ($n=6$)	31 ± 12 ($n=6$)
50	100	48,146 ± 6,933** ($n=6$)	4,763 ± 2,081 ($n=6$)	1,643 ± 367** ($n=6$)	57 ± 19 ($n=6$)

[a] PBMCs were cultured in the presence of Co^{2+} or Ni^{2+} (10–50 µM) and ascorbic acid (20–100 µM). Free radicals were not detectable under the applied conditions.
[b] Lymphocyte proliferation was measured after 4.5 days by [methyl-^{3}H]thymidine incorporation and expressed as mean CPM ± SE.
[c] IFN-γ levels in the supernatants were measured after 4 days with an ELISA method and expressed as mean pg/ml ± SE.
* significantly different from the controls $p<0.05$.
** significantly different from the controls $p<0.01$.

$CoCl_2 \cdot 6H_2O$ or $K_2Cr_2O_7$ (11). Questions arising from false positive patch-test responses to $NiSO_4 \cdot {}_7H_2O$ have been noted by others (12). In that respect, a proposal has recently been put forward by Lachapelle et al. (13) that 5% $NiSO_4 \cdot {}_7H_2O$ in petrolatum, as used in the European standard series, should be lowered to 2.5%, as used in the North American and Japanese standard series.

Metal-ion-mediated free radical generation was detected and identified by ESR spectroscopy with the use of DMPO as a spin trap. Although some limitations of this technique have been noted (4), spin-trapping is a frequently used procedure to create relatively stable radical adducts. Co^{2+} (10–50 μM) generated the same amounts of free radicals in PBMC cultures from patients and controls (Fig. 2). In contrast, under the applied conditions, no free radicals were detectable after addition of Ni^{2+} (10–50 μM). A higher efficacy of Co^{2+} to generate free radicals, in comparison with Ni^{2+}, is in agreement with results obtained in H_2O_2-containing PBS solutions (4) and with *in vivo* results in circulating blood in rats with the use of high concentrations (10–500 mM) of the metal-ions (7). Although ascorbic acid is quickly oxidized in PBMC cultures, the Co^{2+}-generated free radicals were efficiently scavenged by ascorbic acid (4). Higher doses of ascorbic acid were not employed in this study, to avoid the possible effect of metal-ion chelation (18) on Ni^{2+} and Co^{2+} allergenity. In conflict with our expectations, pretreatment of PBMC cultures with ascorbic acid did not influence Ni^{2+}- and Co^{2+}-induced [methyl-^{3}H]thymidine incorporation and IFN-γ production in cultures from allergic subjects (Table I). These results suggest that the formation of antigenic determinants of Co^{2+} and Ni^{2+} is not initiated by metal-ion-generated free radicals. As most often postulated, ACD to Co^{2+} and Ni^{2+} is probably the result of formation of specific antigenic coordination complexes of metal-haptens with electron-rich residues of proteins or other macromolecules (8).

In conclusion, inhibition of Co^{2+} (10–50 μM)-mediated free radical generation with ascorbic acid did not influence cellular [methyl-^{3}H]thymidine uptake and IFN-γ production in PBMC cultures from allergic individuals, and no detectable amounts of free radicals were obtained with Ni^{2+} (10–50 μM). It is therefore unlikely that free radicals are involved in ACD to Ni^{2+} and Co^{2+}.

ACKNOWLEDGEMENTS

This study was supported by grants from the Swedish Foundation for Health Care Sciences and Allergy Research, the Swedish Council for Work Life Research, the Swedish Medical Research Council, the Swedish Association against Asthma and Allergy, and the Hesselman Foundation. The authors would like to thank Anne Svensson for taking the blood samples from the participants of this study, Lizbet Skare and Gunborg Lindahl for their help with patch-testing and Torbjörn Astlind for his expert assistance with the ESR measurements.

REFERENCES

1. Lidén C, Wahlberg JE, Maibach HI. Skin. In: Goyer RA, Klaassen CD, Waalkes MP, eds. Metal toxicology. London: Academic Press; 1995, p. 447–464.
2. Lidén C, Wahlberg JE. Cross-reactivity to metal compounds studied in guinea pigs induced with chromate or cobalt. Acta Derm Venereol (Stockh) 1994; 74: 341–343.
3. Moulon C, Vollmer J, Weltzien HU. Characterization of processing requirements and metal cross-reactivities in T cell clones from patients with ACD to nickel. Eur J Immunol 1995; 25: 3308–3315.
4. Van den Broeke LT, Gräslund A, Nilsson JLG, Wahlberg JE, Scheynius A, Karlberg A-T. Free radicals as potential mediators of metal-allergy: Ni^{2+}- and Co^{2+}-mediated free radical generation. Eur J Pharmaceut Sci, in press.
5. Shi X, Dalal NS, Kasprzak KS. Generation of free radicals from lipid hydroperoxides by Ni^{2+} in the presence of oligopeptides. Arch Biochem Biophys 1992; 299: 154–162.
6. Shi X, Dalal NS, Kasprzak KS. Generation of free radicals from model lipid hydroperoxides and H_2O_2 by Co(II) in the presence of cysteinyl and histidinyl chelators. Chem Res Tox 1993; 6: 277–283.
7. Wang X, Yokoi I, Liu J, Mori A. Cobalt(II) and nickel(II) ions as promoters of free radicals in vivo: detected directly using electron spin resonance spectrometry in circulating blood in rats. Arch Biochem Biophys 1993; 306: 402–406.
8. Basketter D, Dooms-Goossens A, Karlberg A-T, Lepoittevin JP. The chemistry of contact allergy: why is a molecule allergenic? Contact Dermatitis 1995; 32: 65–73.
9. Gäfvert E, Shao LP, Karlberg A-T, Nilsson U, Nilsson JLG. Contact allergy to resin acid hydroperoxides. Hapten binding via free radicals and epoxides. Chem Res Toxicol 1994; 7: 260–266.
10. Schmidt RJ, Khan L, Chung LY. Are free radicals and not quinones the haptenic species derived from urushiols and other contact allergenic mono- and dihydric alkylbenzenes? The significance of NADH, glutathione, and redox cycling in the skin. Arch Dermatol Res 1990; 282: 56–64.
11. Wahlberg JE. Occupational ACD. In: Kimber I, Maurer T, eds. Toxicology of contact hypersensitivity. London: Taylor & Francis Ltd.; 1996. p. 57–74.
12. Al-Tawil NG, Marcusson JA, Möller E. Lymphocyte transformation test in patients with nickel sensitivity: an aid to diagnosis. Acta Derm Venereol (Stockh) 1981; 61: 511–515.
13. Lachapelle JM, Ale SI, Freeman S, Frosch PJ, Goh CL, Hannuksela M, et al. Proposal for a revised international standard series of patch tests. Contact Dermatitis 1997; 36: 121–123.
14. Renström A, Larsson PH, Malmberg P, Bayard C. A new amplified monoclonal rat allergen assay used for evaluation of ventilation improvements in animal rooms. J Allergy Clin Immunol 1997; 100: in press.
15. Al-Tawil NG, Marcusson JA, Möller E. In vitro testing for cobalt sensitivity: an aid to diagnosis. Acta Derm Venereol (Stockh) 1984; 64: 203–208.
16. Sinigaglia F, Scheidegger D, Garotta G, Scheper R, Pletscher M, Lanzavecchia A. Isolation and characterization of Ni-specific T cell clones from patients with Ni-contact dermatitis. J Immunol 1985; 135: 3929–3932.
17. Karttunen R, Silvennoinen-Kassinen S, Juutinen K, Andersson G, Ekre HPT, Karvonen J. Nickel antigen induces Il-2 secretion and Il-2 receptor expression mainly on $CD4^+$ T cells, but no measurable gamma interferon secretion in peripheral blood mononuclear cell cultures in delayed type hypersensitivity to nickel. Clin Exp Immunol 1988; 74: 387–391.
18. Sugiyama M. Role of cellular antioxidants in metal-induced damage. Cell Biol Toxicol 1994; 10: 1–22.