Validity of MELISA® for metal sensitivity testing

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Abstract

OBJECTIVE: This study was carried out to evaluate the reproducibility, sensitivity, specificity, and reliability of the MELISA® Test for detecting metal sensitivity in patients with clinical symptoms of a type IV hypersensitivity to metal.

DESIGN: Blood from 250 patients was tested in MELISA® against up to 20 different metals in 2 to 3 concentrations. The frequency and distribution of metal reactivities, the sensitivity and specificity of nickel reactivity in patients with and without confirmed or suspected sensitivity to nickel, and the roles of lymphocyte concentration and concentration of inorganic mercury were analyzed. In addition, for reproducibility testing, 196 metal tests were performed in duplicate, and intra- and interassay variations of MELISA® results were examined in patients patch-test positive for the relevant metal.

RESULTS: Among the 250 patients, reactivity to 0, 1, 2, 3, 4, or ≥ 5 metals was 26%, 36%, 15%, 12%, 6%, and 5%, respectively. Reactivity was most frequent to nickel (73%), followed by titanium (42%), cadmium (18%) gold (17%), palladium (13%), lead (11%), beryllium (9%), inorganic mercury (8%), tin (8%), and phenylmercury (6%). All patients (n=15) with confirmed or suspected nickel allergy were positive in MELISA®, while patients with no suspicion of nickel allergy were either negative (n=6) or very low positive (n=4) in MELISA® . MELISA® reactivity is directly dependent on lymphocyte concentration: the higher the lymphocyte concentration per test, the stronger the reactivity. Concentrations of inorganic mercury $> 0.5\ \mu$g/ml cause non antigen-specific (mitogenic) reactions in a majority of patients. The reproducibility rate was 94% using a cut-off of Stimulation Index ≥ 3 or 99% using a cut-off of ≥ 5. While the absolute intra- and interassay Stimulation Index values may vary, the qualitative results are highly reproducible.

CONCLUSION: The MELISA® Test is reproducible, sensitive, specific, and reliable for detecting metal sensitivity in metal-sensitive patients.

Abbreviations

cpm	counts per minute
LTT	lymphocyte transformation test
MELISA®	memory lymphocyte immunostimulation assay
PWM	poke weed mitogen
SI	stimulation index

Introduction

Metal sensitivity is conventionally diagnosed with the epicutan or "patch-test" in which the suspected allergizing substance is applied to the skin for 3–4 days and the site subsequently evaluated for erythema, papules or vessicles. While a positive reaction may be indicative of a specific metal allergy, the test is unable to distinguish between allergic and irritative reactions, has a low sensitivity and poor reproducibility, appears to be relevant only for allergens for which skin is the major route of sensitization, and may itself induce *in vivo* sensibilization or exacerbate symptoms in sensitized individuals [1–5]. An alternative is the lymphocyte transformation test (LTT) in which patient lymphocytes (memory cells) are co-cultivated for 5–6 days with the suspected allergen, and the resulting blast transformation and lymphocyte proliferation are evaluated by morphological analysis and ^{3}H-thymidine incorporation, respectively. Originally developed in the mid-sixties for evaluating histoincompatible class II HLA antigens [6,7], the method was modified for class II antigen typing [8] and also applied extensively to detecting type IV allergies to drugs, metabolites, infectious organisms, and metals [9–17]. LTT became a common test for sensitivity to nickel, gold, cobalt, chromium, and palladium [1,18–20]. LTT to berrylium is now accepted as the "gold standard" for diagnosing berrylliose lung disease [21,22].

In 1994 Stejskal et al published a modification of the LTT for detecting metal sensitivity – the MELISA® test (memory lymphocyte immunostimulation assay)[23]. By utilizing a higher number of lymphocytes per test, selecting metal concentrations for non-cytotoxicity and non-mitogenicity, depleting the lymphocyte population of monocytes, and confirming the radiological result with a morphological analysis, the sensitivity and specificity of the assay could be improved. During the last 6 years, several laboratories around the world have been licensed to perform MELISA® testing, and a number of papers demonstrating its clinical utility have since been published [24–26] or are in preparation [27].

The former Laboratory Dr. Schiwara & Partner in Bremen was licensed to perform MELISA® testing in 1999. To fulfill the ISO 17025 accreditation requirements, the test was validated as to sensitivity, specificity, reproducibility, and reliability. In the present study, the results of this validation of MELISA® for metal sensitivity testing are presented.

Material and methods

Blood samples

For all evaluations except reproducibility testing, the blood used was derived from 250 consecutive samples submitted to the former Laboratory Dr. Schiwara & Partner for routine MELISA® testing during the year 2001. Most samples were from patients with clinical symptoms suspicious of a type IV metal allergy.

For reproducibility testing, blood used was derived from samples submitted during the year 2000 in which sufficient lymphocytes were available for replicate testing on the same day by the same or by two different technicians.

All blood samples were submitted in CPDA monovettes (Sarstedt AG & Co., Nümbrecht) or ACD Solution A vacutainer tubes (Becton Dickinson GmbH, Heidelberg) and transported by normal post or by private courier to arrive in our laboratory within 24 hours, at most 48 hours, of drawing. Lymphocytes were isolated immediately upon arrival in the laboratory, and either used in MELISA® directly or stored in medium containing 20% pooled, heat-inactivated human AB serum (Sigma-Aldrich Chemie GmbH, Taufkirche) overnight at 4 °C prior to set up.

The referring physicians were primarily general practitioners, allergologists and dermatologists, environmental physicians or homeopathic doctors, less frequently dentists or psychiatrists. Most had practices in Germany, a small portion were situated in Austria, Belgium, England, France, Greece, Holland, Israel, Italy, Sweden, Switzerland, and USA.

MELISA®

The MELISA® test was performed essentially as previously described [14], with minor modifications. Briefly, lymphocytes were isolated from anticoagulated (instead of defibrinated) blood on Ficoll Histopaque (Sigma-Aldrich Chemie GmbH), washed twice in Medium [RPMI-1640 containing Hepes (Life Technologies GmbH, Karlsruhe), 8 mg/l gentamycin (Sigma-Aldrich Chemie GmbH), and 6.25 mM L-Glutamine (Biochrom AG Seromed, Berlin)], resuspended in Medium containing 20% pooled, heat-inactivated human serum, incubated at 37 °C with 5% CO_2 for 30 minutes in a plastic cell culture flask (first monocyte depletion), and resuspended in Medium plus 10% pooled, heat-inactivated human serum (10% Medium) to a concentration of 1×10^6 lymphocytes/ml. One ml cells were then pipetted into the wells of a 24-well (instead of 48-well) cell culture plate (Dunn Labortechnik GmbH, Asbach) pre-coated with metal solutions in 2 to 3 concentrations, and the plates were incubated for 5 days at 37 °C with 5% CO_2. Three negative controls (only lymphocytes in 10% Medium) and 1 positive control [lymphocytes in 10% Medium plus PWM (Poke Weed Mitogen, Sigma-Aldrich Chemie GmbH)(instead of Purified Protein Derivative)] were included in each test. After 5 days, 600 μl of cell suspension from each well was transferred to a new 24-well plate (second monocyte depletion) and the cells pulsed for 4 hours

with 3 μC methyl-^{3}H-thymidine (Amersham Buchler GmbH & Co. KG, Braunschweig), specific activity 185GBq/mmol. The cells were harvested (Inotech Cell Harvester, Wallac Distribution GmbH, Freiburg) onto filter paper, the filter paper dried in a microwave oven (instead of at room temperture overnight) and the radioactivity measured in a liquid scintillation counter (1450 Microbeta Trilux, Wallac Distribution GmbH).

A positive reaction was defined as a Stimulation Index (SI) $\geq$ 3 as calculated below:

SI = cpm in test well/average cpm in negative control wells.
A SI between 2 and 3 was interpreted as a "possible sensibilization", and a SI < 2 was considered negative.

Cells from the 5-day cultures were additionally analysed morphologically after staining cytospin preparations with Rapid Differential Hematology Staining solutions (Dade Behring AG, Marburg). Only tests in which the radioactively positive results showed the presence of lymphoblasts and radioactively negative results showed only viable, small lymphocytes (non-cytotoxicity and non-stimulation) were accepted as valid.

Table 1: Duplicate MELISA® testing of a single patient

Antigen	Test 1		Test 2	
	cpm	SI	cpm	SI
+ Control	421,481	240.0	275,182	136.6
– Control	1,756	1.0	2,030	1.0
Be 1	1,508	0.9	1,691	0.8
2	1,311	0.8	1,676	0.8
Pb 1	1,394	0.8	1,615	0.8
2	2,339	1.3	1,797	0.9
Cd 1	1,816	1.0	1,492	0.7
2	989	0.6	1,165	0.6
Au 1	1,083	0.6	1,014	0.5
2	1,050	0.6	1,210	0.6
Ni 1	**7,580**	**4.3**	**6,472**	**3.3**
2	**34,544**	**19.7**	**23,153**	**11.4**
Pd 1	1,063	0.6	1,443	0.7
2	934	0.5	1,669	0.8
HgCl 1	1,483	0.8	2,413	1.2
2	1,720	1.0	1,958	1.0
HgMe 1	1,340	0.8	1,640	0.8
2	1,360	0.8	1,274	0.6
Ti 1	2,763	1.6	2,489	1.2
2	1,125	0.6	1,804	0.9
Sn 1	1,383	0.8	2,188	1.1
2	1,664	1.0	2,309	1.1

– control: cpm value shown is the average of 3 replicates
1,2: refers to two concentrations of the metal indicated

Table 2: Day-to day variation in MELISA® testing

Antigen	Test 1	Test 2	Test 3	Test 4	Test 5
HgCl (SI):	38.0	48.6	38.8	24.4	21.3
+ Control (SI):	30.8	62.0	68.5	47.0	39.8
– Control (cpm):	2,392	2,732	1,835	2,733	2,190

– Control: cpm value shown is the average of 3 replicates

Table 3: Replicate testing (n = 196)

		Test 1	
		+	–
Test 2	+	32	0
	–	12	152

Table 4: Discordant results (n=12)

Positive (SI $\geq$ 3)	Negative (SI <3)
3.0	1.7
3.1	0.6
3.2	1.3
3.4	1.0
3.4	1.5
3.5	2.1
3.7	2.4
3.7	2.8
4.0	2.1
4.3	2.9
4.6	2.8
10.1	1.8

Results

Reproducibility. MELISA® reproducibility was evaluated in three ways:

First, blood from patients was tested in duplicate on the same day by two different technicians. Typical data are shown in Table 1 for Patient 1, who is patch-test positive for nickel. While the actual SI values vary slightly between replicates, the qualitative results are clearly concordant, showing positive reactivity for nickel and a negative response for all other metals tested.

Second, blood from Patient 2 (patch-test strongly positive for mercury) was tested on five different days in two- to four-week intervals (Table 2). Again, despite variation in the actual SI values, this patient is reproducibly positive for inorganic mercury (mean SI = 34.2 ± 11.2). Results with ethyl-/methyl-/ and pheny-mercury were negative (SI $\leq$ = 1.3, data not shown), demonstrating specificity for the inorganic form. The background proliferation (negative control values)

Table 5: Role of lymphocyte concentration

cells/ml	Hg (SI)	PWM (SI)	Ni (SI)	PWM (SI)
1,000,000	**21.3**	**39.8**	**41.1**	**104.8**
500,000	**4.4**	**45.6**	**26.0**	**154.3**
250,000	0.5	**61.5**	**13.0**	**98.5**
125,000	0.6	**11.5**	**7.3**	**131.3**
62,500	1.0	**15.0**	2.9	**147.2**
31,250	1.2	**6.3**	1.4	**50.5**
15,625	1.3	**5.7**	0.8	**10.4**
7,813	1.0	2.3	2.1	1.8

PWM = poke weed mitogen (positive control)

Table 6: Distribution of MELISA® reactivities to titanium and nickel in 250 patients

	Ti	Ni
SI range	%	%
3–5	35.7	20.8
5–10	42.9	28.9
> 10	21.4	50.3

Table 7: Nickel sensitivity and specificity

Nickel allery confirmed/suspected	SI	Sex
+	31.0	F
+	24.4	F
+	21.9	F
+	19.3	F
+	18.2	F
+	18.2	F
+	17.3	F
+	15.4	F
+	13.5	F
+	12.9	F
+	12.9	F
+	9.5	F
+	8.4	F
+	8.2	F
+	8.0	F
–	6.4	M
–	6.3	M
–	5.8	M
–	4.3	M
–	1.9	M
–	1.9	M
–	1.4	F
–	1.2	M
–	1.0	F
–	0.8	F

remained constant (mean cpm = 2376 ± 381). Similar results were obtained from a patient patch-test positive for nickel (data not shown).

Finally, a total of 196 metal tests were performed in duplicate on identical days by the same or different technicians (Table 3). The results show a concordance rate of 94%. In eleven of the 12 discordant results, the positive result had a low SI of ≤ 4.6 (Table 4). The reproducibility rate was, therefore, 94% using a cut-off of SI ≥ 3 or 99% using a cut-off of SI ≥ 5.

Role of lymphocyte concentration. To evaluate the effect of lymphocyte concentration on MELISA® results, serial dilutions of cells from Patient 2 (described above) and Patient 3 (patch-test positive for nickel) were tested against mercury and nickel, respectively (Table 5). In both cases, metal-specific reactivity decreased rapidly, becoming negative at concentrations of 250,000 cells/ml (mercury) and 62,500 cells/ml (nickel), while the non-specific mitogenic reactivity remained positive in both cases until the cell concentration was 7,813 cells/ml. The background proliferation was less affected by the cell concentration, ranging from 2698 to 964 cpm (average: 1048 cpm) for mercury and from 2823 to 678 cpm (average: 1398 cpm) for nickel (data not shown).

Role of metal concentration. To evaluate the reported mitogenic activity of high concentrations of inorganic mercury (> 0.5 µg/ml), lymphocytes from 10 randomly selected patients were tested with serial dilutions of HgCl (Figure 1). Concentrations of HgCl of > 0.5 µg/ml induced positive stimulation in eight out of ten patients (80%); only one of these patients showed a weak response (SI = 3.2) to concentrations of ≤ 0.5 µg/ml.

Frequency of metal reactivity. In the patient group tested (n = 250), 26% were negative to all metals tested (up to 20), 36% were positive to 1 metal, 15% to 2 metals, 12% to 3 metals, 6% to 4 metals and 5% to 5 or more metals (Figure 2).

Most frequent metal reactivities. The 10 metals to which the 250 patients most frequently responded were nickel (73%), titanium (42%), cadmium (18%), gold (17%), palladium (13%), lead (11%), beryllium (9%), inorganic mercury (9%), tin (8%), and phenylmercury (6%) (Figure 3). Reactivity frequencies of < 3.6% were found to aluminum, chromium, copper, ethylmercury (Thimerosal), indium, methylmercury, platinum, and silver. No reactivity was found to molybden and cobalt.

To facilitate comparison to published data, the MELISA® reactivites to titanium and nickel were further analyzed according to "weak sensitivity" (SI 3–5), "sensitivity" (SI 5–10) or "strong sensitivity" (SI > 10) (Table 6). While one-third of titanium results showed "weak sensitivity" and only one-fifth showed "strong sensitivity", one-fifth of nickel results showed "weak sensitivity" but 50% showed "strong sensitivity".

Nickel sensitivity and specificity. Fifteen persons with confirmed (patch-test positive) or suspected (dermal sensitivity to jewelry or jeans buttons) nickel allergy and 10 persons with no evidence of nickel allergy were tested in MELISA® (Table 7). All 15 persons with confirmed or suspected nickel allergy were MELISA® positive (SI 8.0 – 31.0); all were female. Of those with no suspicion of nickel allergy, 6 were negative (SI ≤ 1.9) and 4 showed a low positive reactivity (SI 4.3 to 6.4).
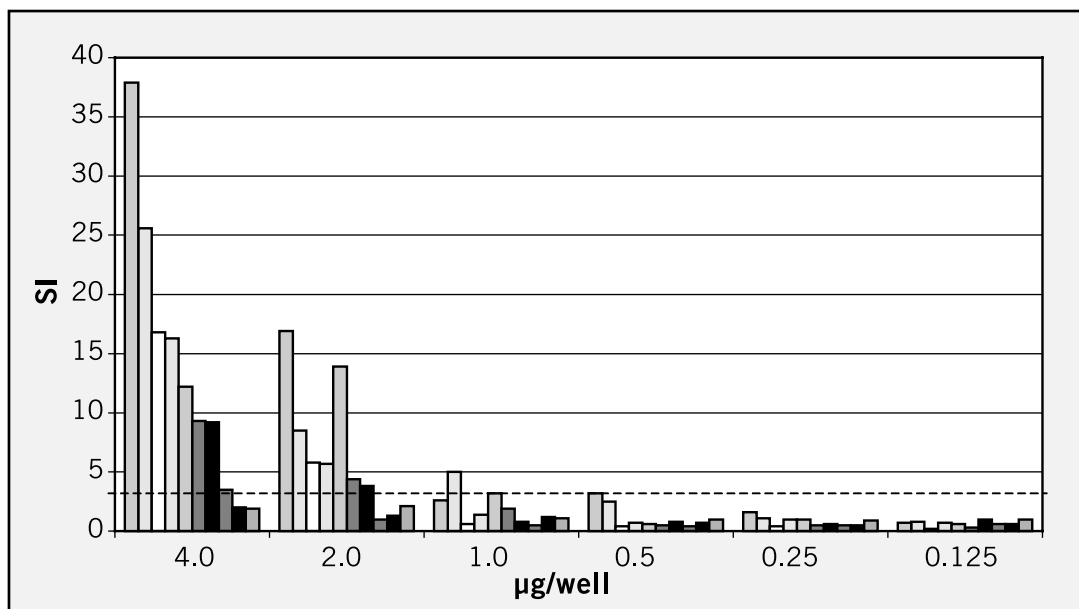
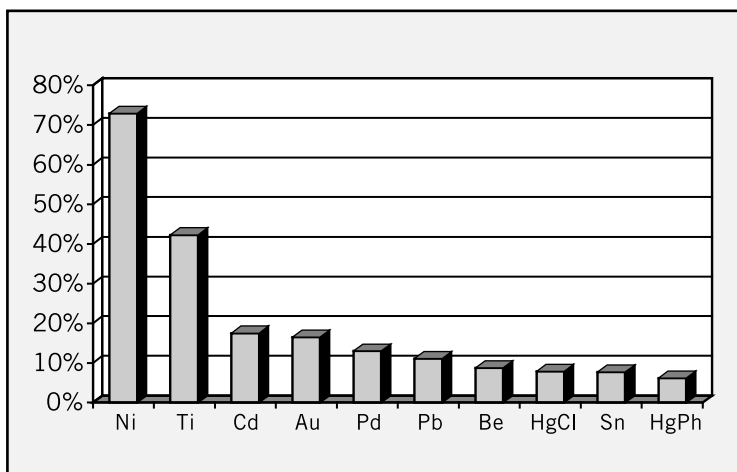**Fig. 1:** Reactivity to various concentrations of HgCl

Fig. 3: Ten most frequent metal reactivities in 250 patients
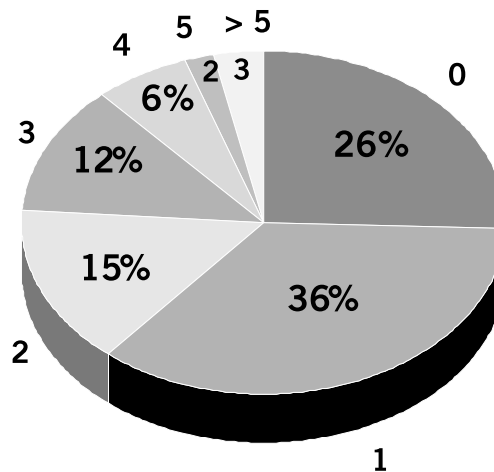

Fig. 2: Frequency of response to various numbers of metals (n = 250)

Discussion

Since their introduction forty years ago, lymphocyte proliferation assays have been utilized as diagnostic tools in the clinical evaluation of T cell hypersensitivity to drugs, metabolites, and metals, as well as for the detection of antigen-specific cellular reactivity in severely immunocompromised patients [9–17]. Their more widespread application and acceptance, however, has been hampered by the limited and sometimes conflicting data available on their reliability [17,28,29]. In this study we evaluate the reproducibility, sensitivity, specificity, and reliability of one such proliferation assay – MELISA® – first published in 1994 [23] and introduced into our laboratory in 1999. This evaluation was peformed in conjunction with the accreditation of our laboratory (ISO 17025) in 2001.

As with any complex biological assay, MELISA® is dependent on the systematic fine-tuning of a number of factors, e.g., cell concentration, metal concentration,

culture conditions, media supplements, all of which contribute to considerable technical variation. For that reason a demonstration of acceptable intra- and inter-assay reproducibility is critical. The data presented here show a high qualitative intra- and inter-assay concordance of results and an overall reproducibility rate of 94% using the standard cut-off of SI ≥ 3.0. The 6% discordant results were (with one exception) in a low-positive range (SI ≤ 4.6), such that a small proportion of MELISA® results in this range might be considered to be in the "gray zone" characteristic of most diagnostic assays. Where clinically relevant, such results should be confirmed by repeat testing. If a cut-off of SI ≥ 5.0 is applied as reported in some MELISA® studies [24], the reproducibility rate is 99%. Remarkably, there was no difference in reproducibility when duplicate testing was peformed by the same or by two different technicians (personal observation). Mroz et al reported con-

cordant results in 9 out of 10 samples split and tested in two different laboratories for reactivity to beryllium in LTT [21].

To our knowledge, the data in the present study represent the most extensive reproducibility evaluation of an LTT published to date.

The use of a higher number of lymphocytes in MELISA® ($1x10^6$ cells/test) compared to conventional LTTs (100,000 to 250,000 cells/test) has been claimed to increase its sensitivity [23]. The data presented here with serial dilutions of lymphocytes from both a mercury-sensitive and a nickel-sensitive patient support this claim. Antigen-specific reactivity results from proliferation of a small proportion of memory cells and is consequently directly dependent on the number of lymphocytes assayed. In contrast, the polyclonal proliferative response to mitogens (e.g., PWM) is much less dependent on cell number. Mroz et al reported improved sensitivity to beryllium in an LTT using 250,000 lymphocytes/test compared to published results with 100,000 lymphocytes/test [21]. Compared to MELISA®, LTTs utilizing $<1x10^6$ cells/test will show reduced sensitivity and a consequent high percentage of false-negative results.

Not only lymphocyte concentration but also metal concentration can affect results in lymphocyte proliferation assays. Early LTTs for metals, e.g. inorganic mercury, demonstrated non-specific stimulation in non-sensitized individuals [28,29]. This effect was subsequently shown to be due to the use of high, mitogenic concentrations of inorganic mercury, *i.e.*, >0.5 $\mu g/ml$ [14]. The data in this study on 10 patients tested with a serial dilution of inorganic mercury confirm this observation. Mercury-specific sensitivity can be concluded only from positive reactivity with concentrations of ≤ 0.5 $\mu g/ml$ (as used for all other tests in this study). Similarly, high concentrations of nickel (≥ 10 $\mu g/ml$) have been reported to induce non-specific proliferation [18]. Nickel concentrations used in this study were ≤ 5 $\mu g/ml$.

As the 250 patients in the present study all had clinical symptoms suspicious of metal allergy, it is not surprising that the majority (74%) were positive to one or more of the up to 20 different metals tested. In a study of 930 patients in Södertälje, Sweden, 62% were positive in MELISA® to one or more metals [24]. The lower figure may be due to the use of a higher cut-off (SI ≥ 5.0) and fewer metals tested (n=15) than in the present study. Even lower results were obtained in a report on ca. 5,000 patients tested with a modified MELISA® – the so-called LTT-CITA®: 58% [30]. This reduced sensitivity is most probably a consequence of the low number of cells employed (250,000/test) coupled with the limited number of metals tested (n=10). The 26% of patients in the present study with apparent clinical symptoms of metal allergy but negative to all metals tested may have hypersensitivity to a metal not tested, may be suffering from metal toxicity (not detectable with MELISA®), or may have an illness with no metal etiology.

In all studies to date, whether performed with patch-testing or with lymphocyte proliferation assays, the most frequently sensitizing metal has been found to be nickel, more often in females than in males [18,24–26]. In this study, too, the highest rate of reactivity was with nickel (73%). The rates reported for three independent groups using the same MELISA® assay but with a cut-off of SI ≥ 5.0 were variable but all lower: ca. 22% from Munich, ca. 36% from Södertälje, and ca. 47% from Uppsala [24]. If the 20.8% of nickel responses falling in the range of SI 3–5 are deducted from the total nickel reactivity in our study (73%), the resulting reactivity level (57.8%) is still somewhat higher than that reported for Uppsala. Approximately 28% of patients responded to nickel in the less sensitive LTT-CITA® [30].

To further analyze the sensitivity and specificity of the nickel reactivities in this study, patients with and without suspicion of nickel allergy were tested in MELISA®. While the sensitivity was 100%, the specificity appeared to be lower: four persons with no suspicion of nickel allergy showed low positive nickel reactivity (SI 4.3–6.4). Patch-test results on these patients are not available. Repeat MELISA® testing was not possible. The patient with the highest SI in this group [6.4] has diabetes, psoriasis, and food allergies. While these 4 patients may have false-positive nickel results, they may also have low-level, asymptomatic nickel sensitivity. All four are men, who presumably wear little jewelry and may, therefore, fail to notice dermal sensitivity. In addition, nickel-reactive T cells have been reported in persons with a negative patch-test and no history of contact dermatitis [31].

A major difference in reactivity to titanium was found in our study (42%) compared to the MELISA® studies of Munich (ca. 1.5%), Södertälje (ca. 6%), and Uppsala (10%) [24]. Even after deducting the titanium responses in the range of SI 3–5 in our data (35.7%) to facilitate comparison with the above-mentioned studies using a higher cut-off, the resulting rate (27%) is still considerably higher. A mitogenic concentration of titanium is unlikely as an explanation as, first, such a concentration of titanium has never been reported, second, the same concentrations of titanium were tested in our study as in the three reported studies, and, third, the majority of patients in our study (58%) did not respond to titanium. The higher rate in our study might be due to the higher rate of exposure (*i.e.* dental titanium implants) in our patients presenting in 2001 compared to those presenting in 1996/1997 [24]. A correlation with patch-test results is not possible as patch-testing for titanium is generally not peformed. While the true extent of titanium sensitivity remains to be clarified, the titanium reactivities reported here are reproducible, correlated (where clinical data are available) with titanium exposure (cosmetics, dental implants, orthopedic protheses), and are clinically relevant, *i.e.*, decrease in titanium-positive patients following reduction of titanium exposure and improvement of clinical symptoms [27].

In the present study, reactivity to inorganic mercury was somewhat lower (8%) than that reported for Munich (ca. 14%), Södertälje (ca. 21%), and Uppsala (ca. 33%)[24] but comparable to that found in the group tested with LTT-CITA® (8%)[30]. The higher rates in the Swedish groups may be due to the deliberate inclusion of a number of patients who were patch-test positive for mercury, the higher referral rate of patients from dentists, and the higher rate of patients with amalgam fillings (VDM Stejskal, personal communication). Recent MELISA® testing for inorganic mercury in 29 Italian patients and 42 Swiss patients revealed 14% and 21% positive rates, respectively (Valentine-Thon, unpublished data, 2002). Clearly, mercury-sensitivity rates will vary depending on inorganic mercury exposure primarily from dental amalgams [32].

For the remaining metals reported in this study, comparable levels of reactivity were found in the studies from Munich, Södertälje, and Uppsala performed with MELISA® [24] as well as in the study performed with LTT-CITA® [30].

In conclusion, the data in this study support a high reproducibility, sensitivity, specificity, and reliablity of MELISA® for metal sensitivity testing. The clinical utility of this lymphocyte proliferation assay has been published [24–26]; further reports are in preparation [27].

Acknowledgments

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