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ORIGINAL ARTICLE

Metal-specific lymphocytes: biomarkers of sensitivity in man

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Abstract

Many patients attribute their health problems to amalgam and other dental metals. In genetically susceptible indviduals, mercury and gold may function as haptens and elicit allergic and autoimmune reactions. The frequency of metal-induced lymphocyte responses was examined in 3,162 patients in three European laboratories using MELISA®, an optimized lymphocyte proliferation test. The patients suffered from local and systemic symptoms attributed to dental restorations. The effect of dental metal removal was studied in 111 patients with metal hypersensitivity and symptoms resembling Chronic Fatigue Syndrome (CFS). After consultation with a dentist the patients decided to replace their metal restorations with non-metallic materials. The changes in health and *in vitro* lymphocyte reactivity were studied by inquiries and follow-up MELISA®. Lymphocyte reactivity was also analyzed in 116 healthy subjects with no complaints of metal allergy. A significant number of patients had metal-specific lymphocytes in the blood. Nickel was the most common sensitizer, followed by inorganic mercury, gold, phenylmercury, cadmium and palladium. As compared to lymphocyte responses in healthy subjects, the CFS group had significantly increased responses to several metals, especially to inorganic mercury, phenylmercury and gold. Following dental metal removal, 83 patients (76%) reported long-term health improvement. Twenty-four patients (22%) reported unchanged health and two (2%) reported worsening of symptoms. Following dental metal replacement, the lymphocyte reactivity to metals decreased as well. We propose that an inflammatory process induced by metals may modulate the hypothalamic-pituitary-adrenal axis (HPA axis) and trigger multiple non-specific symptoms characterizing CFS and other chronic conditions like myalgic encephalitis (ME) and multiple chemical sensitivity (MCS).

Abbreviations

CFS	chronic fatigue syndrome
HPA	hypothalamic-pituitary-adrenal
MCS	multiple chemical sensitivity
ME	myalgic encephalitis
MELISA®	memory lymphocyte immuno stimulation assay
PPD	purified protein derivative (Tuberculin)
SI	stimulation index

Introduction

During the last century there has been an increasing interest in the possible harmful effects of dental amalgam. Silver amalgam fillings contain about 50% inorganic mercury together with copper, tin, silver, zinc, and palladium. The toxic effects of mercury, a potent mitochondrial inhibitor are well documented [1]. However, since mercury and other dental metals such as gold and silver also bind to proteins, they may function as haptens and elicit allergic and autoimmune reactions.

Immunological reactions induced by metals are dependent on genetic haplotype and inorganic mercury and gold can induce autoimmunity in genetically susceptible rats or mice [2]. The role of genetics in metal-induced side effects is difficult to study in humans since, with the exception of monozygous twins, subjects with identical genetic background are not available.

One of the ways to study the possible role of metals in the pathogenesis of various degenerative diseases is screening of exposed populations for markers of susceptibility. T-lymphocytes play a crucial role in the induction of all types of immunological reactions and are therefore useful for this purpose [3].

Metals, such as inorganic mercury or nickel, may induce both antigen-specific responses and nonspecific mitogenic responses when added to peripheral blood lymphocytes *in vitro* [4–6]. In order to use the lymphocyte proliferation test as an indicator of metal-induced allergy, it was necessary to modify the test in such a way that only antigen-specific responses were operating. The test is called MELISA®, an acronym for *Memory Lymphocyte Immuno Stimulation Assay* [7]. With MELISA®, it is possible to screen a large number of individuals claiming to suffer from local or systemic side-effects triggered by components of dental amalgam and other metal alloys such as palladium-containing gold alloys. Additional sources of metals are metal pigments such as titanium dioxide ($Ti0_2$, E171), a white coloring agent used in many composites, dental cements, and root fillings [8]. Phenylmercury, an organic mercury preservative, has been used together with lead and arsenic as a component of certain root fillings. We also studied the reactivity of lymphocytes to Thimerosal (Merthiolate, Thiomersal), an ethylmercury-based salt widely used as a preservative in nose and eye drops, vaccines, and in cleaning solutions for soft contact lenses. Finally, the reactivity to nickel, clinically the most common sensitizer, was also studied.

Materials and Methods

Patients

One German and two Swedish laboratories performed a comparative study. The majority of patients (a total of 3,162 patients) were referred for testing by dentists or physicians because of suspected metal allergy. In general, the patients suffered from multiple chronic symptoms typical of Chronic Fatigue Syndrome (CFS). Patient characteristics are shown in Table 1.

A subset of patients referred to the Department of Clinical Metal Biology in Uppsala entered a longitudinal study. A total of 111 patients, 99 females (mean age 47 years, range 17–74 years) and 12 males (mean age 46 years, range 34–60 years) were tested with MELISA® at the beginning of the study. The majority of patients (77%) fulfilled the criteria for CFS [9]; the remaining ones lacked one of the major criteria, or several of the minor criteria. However, both groups are presented as a CFS-like group. Routine laboratory findings were usually within the normal range, but most of the patients reported clinical intolerance to metals. Following consultation with a dentist, most patients decided to replace metal restorations with non-metallic materials such as composites and ceram-

Table 1.

Total number of patients		3162	
SÖDERTÄLJE	Total	Females	Males
Number of patients	930	700 (75%)	230
Age, mean	47.5	48.3	45.2
Age, median	48.0	48.0	46.0
UPPSALA			
Number of patients	1,112	799 (72%)	313
Age, mean	49.0	49.9	46.7
Age, median	49.0	51.0	47.5
MUNICH			
Number of patients	1,120	771 (69%)	349
Age, mean	39.7	41.4	37.5
Age, median	38.0	38.0	37.0

ics. The patients were also prescribed antioxidants, i.e. vitamin and mineral supplements, to counteract oxidative stress related to increased metal exposure during the dental treatment. In some patients who tolerated gold and titanium constructions, only dental amalgam was removed. The patients were contacted after completion of the removal of incompatible dental materials and asked to assess their current health as compared to the pre-treatment situation. At the end of the study, more than 50% of the patients had not been exposed to incompatible dental materials for two years or more.

The control group was comprised of 116 healthy individuals: 75 females (mean age 44 years and range 18–78 years) and 41 males (mean age 44 years and range 17–79 years). During the first years of study controls were recruited by advertising for healthy subjects. Nineteen consecutive subjects, enrolled in the study during 1990, were patch tested with True test and dental screening test. Patch test, performed after MELISA®, showed that five out of 19 subjects (26%) were patch test-positive, mostly against nickel and Thimerosal. The inclusion of metal-sensitive subjects as "controls" could bias the study. Therefore, prospective controls were asked to complete a questionnaire regarding oral and general health, metal exposure and suspected or known metal sensitivity. Individuals reporting oral problems such as gingivitis, burning or skin problems such as itching or dermatitis were excluded from the study. The other exclusion criteria were chronic treatment with drugs, allergy to foods, cosmetic products, pollen, animal allergens or drugs. Yet another exclusion criteria were systemic effects such as urticaria or flu-like symptoms following dental treatment. The elected controls reported no side-effects after metal exposure (for example intrauterine device or metal earrings) and were exposed to dental amalgam at the time of the study or in the past. Many control subjects had gold restorations and root fillings. Participation in the study did not occur until informed consent was obtained.

MELISA®

Lymphocyte reactivity to metals was assessed by the uptake of tritiated thymidine as described previously [7, 10, 11]. Lymphocytes were separated on a Ficoll-Paque gradient and the monocyte content was reduced by adherence to plastics. Non-adherent cells were recovered and diluted to 0.5–1.0 x 10^6 cells per ml in RPMI 1640 medium containing 10 m*M* Hepes, enriched with 10% inactivated normal human AB serum, 8 μg/L gentamycin and 4 m*M* L-glutamin. The cells were cultured in 48-well tissue culture plates, which were coated with different concentrations of metal salts. Twofold dilutions were used for each metal salt per patient. After five days at 37°C and standard culture conditions, new 48-well plates were supplemented with 3 μCi methyl-^{3}H-thymidine (Amersham, England; spec. act. ~3.2 TBq/mmol) per well and 600 μl of cell suspension from each cell culture was added. Cells were harvested in an automatic cell harvester after

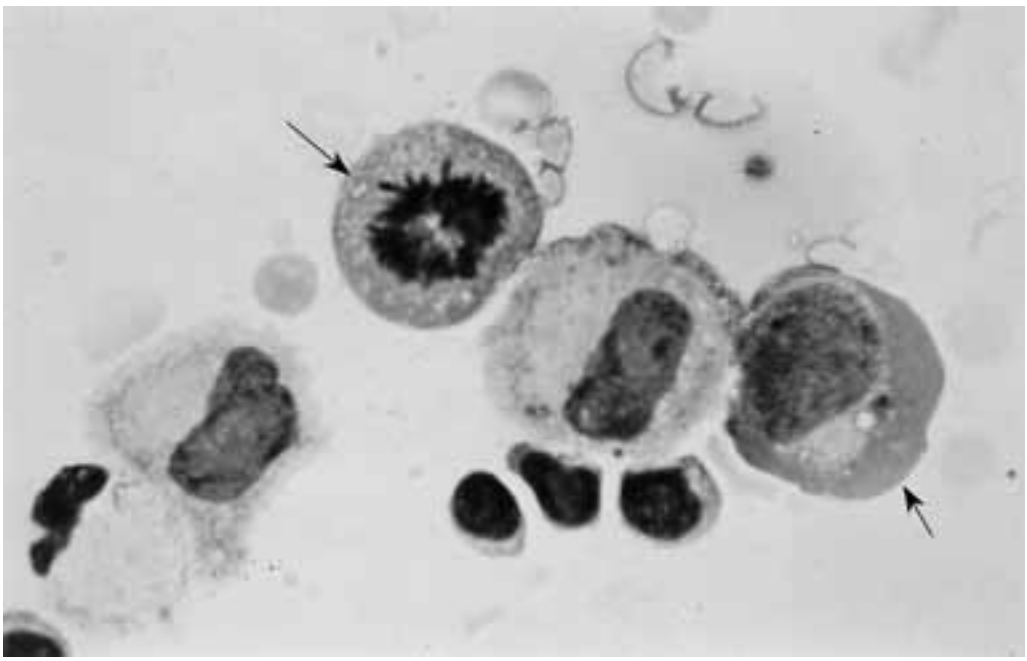

Fig. 1. Metal-induced lymphocytes (lymphoblasts). 2 macrophages and 3 small lymphocytes are also present.

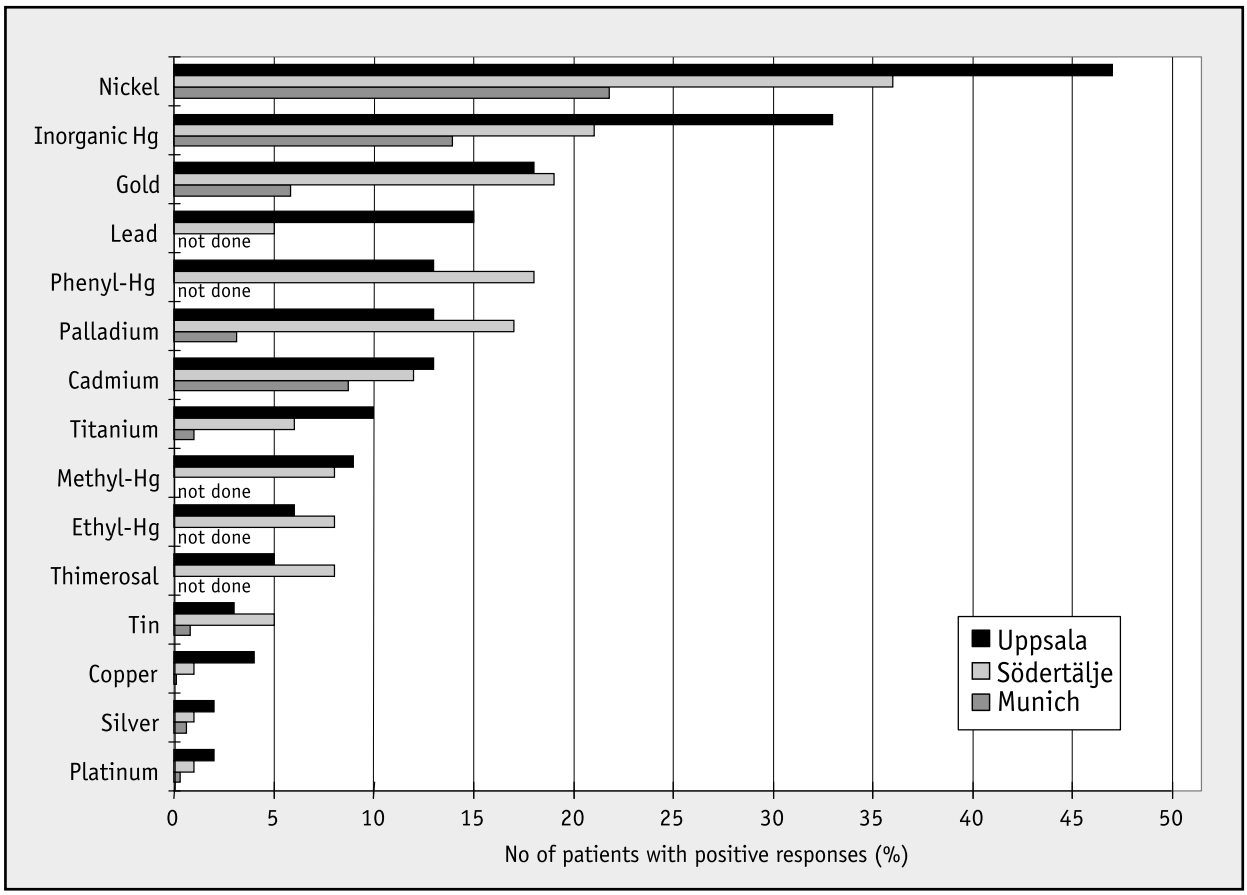

Fig. 2. Prevalence of positive responses to metals in patients tested by three laboratories.

4-hr incubation at 37°C and the radioactivity was measured using a scintillation counter. The increase in ^{3}H-thymidine incorporation in antigen-treated cultures was expressed as a Stimulation Index (SI) which is defined as the activity (cpm) in antigen-treated cultures divided by the mean activity (cpm) in untreated control cultures. Lymphocyte responses induced by metals were characterized by the mean of the two highest responses obtained with each metal salt. Previously, a positive lymphocyte response was defined as SI ≥3, accompanied by an increased number of lymphoblasts in metal-treated cultures [7, 11 and Fig. 1]. In this study, however, the morphological aspect was not taken into consideration. Consequently, we considered SI-values equal to or more than five as positive responses.

Results

The prevalency of positive responses to metals is shown in Fig. 2. Nickel was the most common sensitizer, followed by inorganic mercury, gold, phenylmercury, palladium and lead. Other metals, such as tin, copper, silver and platinum only rarely stimulated lymphocytes.

Lymphocyte responses to inorganic and organic mercury

The results in 3,162 patients indicate that lymphocyte reactivity to low concentrations of inorganic mercury is relatively common. Totally, 719 patients (23%) had mercury-specific lymphocytes in the blood (Fig. 2).

Eighteen and 13%, respectively, of the patients tested in the two Swedish laboratories reacted to phenylmercury. Generally, phenylmercury-specific lymphocytes did not respond to methylmercury or ethylmercury. However, parallel sensitization to several mercurials occurred in many patients.

Fewer than 10% of the patients tested reacted to Thimerosal. Lymphocyte responses induced by Thimerosal were virtually identical to responses induced by ethylmercury. Another organic mercurial, methylmercury, induced positive responses in 8% of the patients tested in Södertälje and in 9% of the Uppsala patients.

When the data was grouped by gender, it became apparent that females responded more frequently to nickel and gold than males (Fig. 3).The frequency of positive responses to inorganic mercury and organic mercury were similar in both sexes.

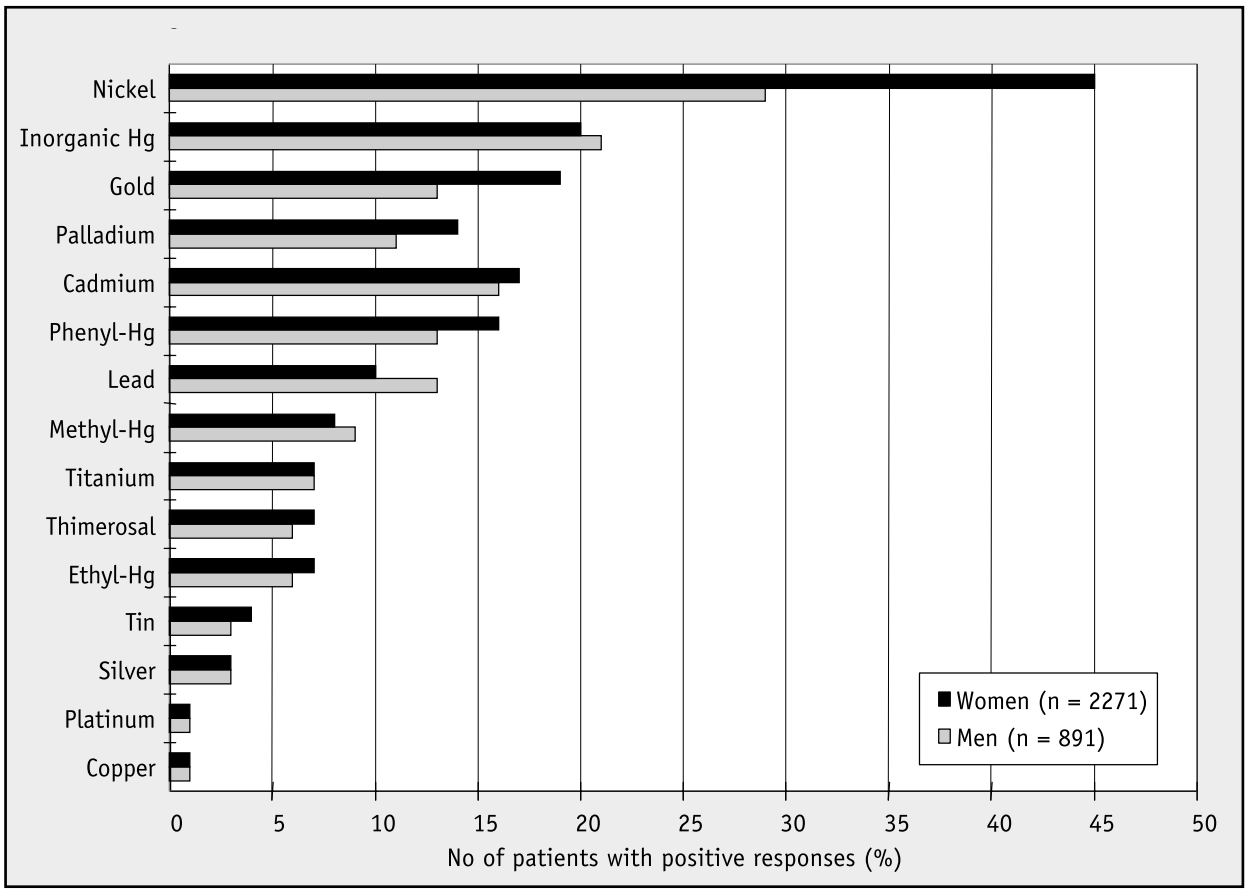

Fig. 3. Prevalence of positive responses to metals in patients stratified by gender (*N*=3162).

Multiple sensitization induced by metals

As seen in Fig. 4, 577 patients (62%) of 930 patients tested in one of the Swedish laboratories reacted to one or several metals. Reactivity to one metal only was found in 233 patients (25%), while 84 patients (9%) reacted to four metals or more; the results were similar in the other two laboratories (data not shown).

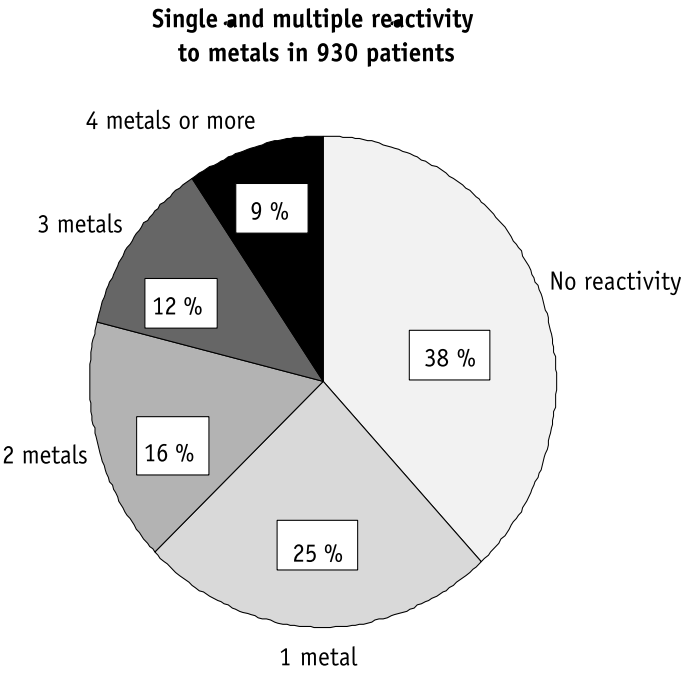

Fig. 4. Single and multiple reactivity to metals in 930 patients.

Reactivity to metals in CFS-like group and in control subjects

The results of MELISA® in 111 patients and 116 controls are shown in Fig. 5. Highly significant differences ($p<0.001$) between the two groups were obtained for the following metals: inorganic mercury, phenylmercury, palladium and gold. In addition, significant responses ($P<0.01$) were obtained for cadmium, titanium, lead, and palladium. The difference in responsiveness to methylmercury and silver was also significant ($P<0.05$). There was no significant difference among the responses in control cultures, responses to PPD, and those induced by other metals. It should be pointed out that the statistical

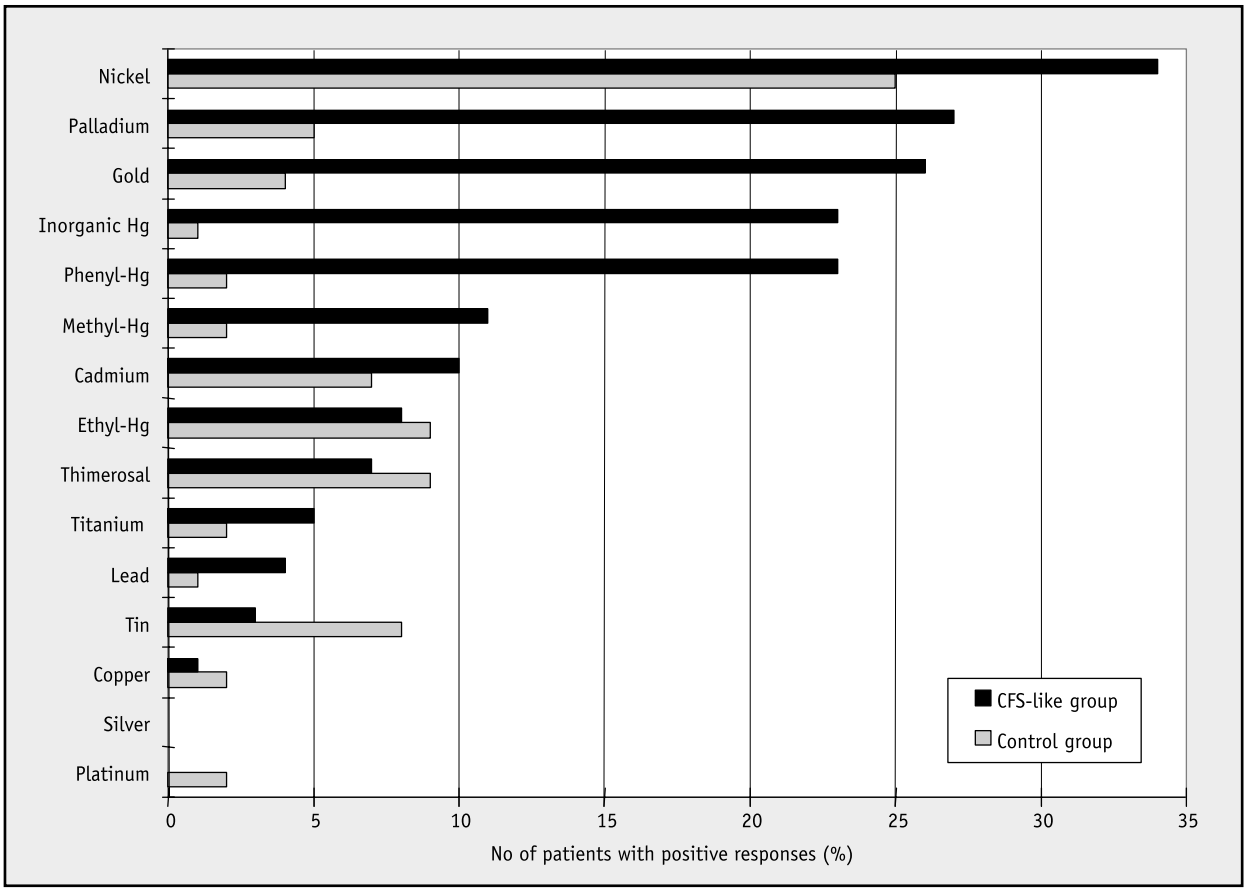

Fig. 5. Prevalence of positive responses to metals in CFS-like group and control group.

evaluation was performed comparing the mean stimulation index-values (SI-values) of the entire CFS (n=111), and control groups (n=116). At the end of the study, 98 patients out of 111 had completed removal of incompatible dental materials. Eighty-three patients (76%) reported long-term health improvement, 24 patients (22%) reported unchanged health status or improvement of some, but worsening of other symptoms, and two patients (2%) reported a worsening of symptoms.

The results of the follow-up MELISA® compared to MELISA® performed at the beginning of the study, in 73 patients who agreed to repeated testing, are shown in Fig.6. There was a marked decrease in lymphocyte reactivity to inorganic mercury, as well as to other metals present in metal alloys as compared to the results obtained at the start of the study. In contrast, the reactivity to nickel did not change substantially.

Discussion

A significant number of patients had metal-specific lymphocytes in the blood. Nickel was the most frequent sensitizer, followed by inorganic mercury, phenylmercury, gold, cadmium, palladium and lead. Nickel sensitization is dependent on the wearing of nickel-containing earrings. Females have a higher rate of sensitization than males [12, 13] which is confirmed at the lymphocyte level. Interestingly, lymphocyte responses induced by inorganic and organic mercury were sex-independent, implying the role of mercury from dental amalgam and other external sources as a cause of sensitization.

The high prevalence of gold and palladium-specific responses is not surprising when considering results of patch tests [14, 15]. In a recent study of 397 patients claiming intolerance to dental metal alloys, 23% demonstrated patch test positivity with gold sodium thiosulfate; the prevalence of positive tests to palladium was 8% [14]. Yet in another study the frequency of gold-positive skin reactions in 832 patients was 8.6% [15]. In a Finnish study of 52 patients with gold-positive patch test, 75% of patients were positive in the lymphocyte proliferation test [16].

The similar frequency of lymphocyte responses to Thimerosal and ethylmercury reflects the fact that ethylmercury is the main allergenic epitope of Thimerosal, a salt of ethylmercury thiosalicylate [17]. Methylmercury elicited lymphocyte responses in 8–9% of Swedish patients tested. This is the first

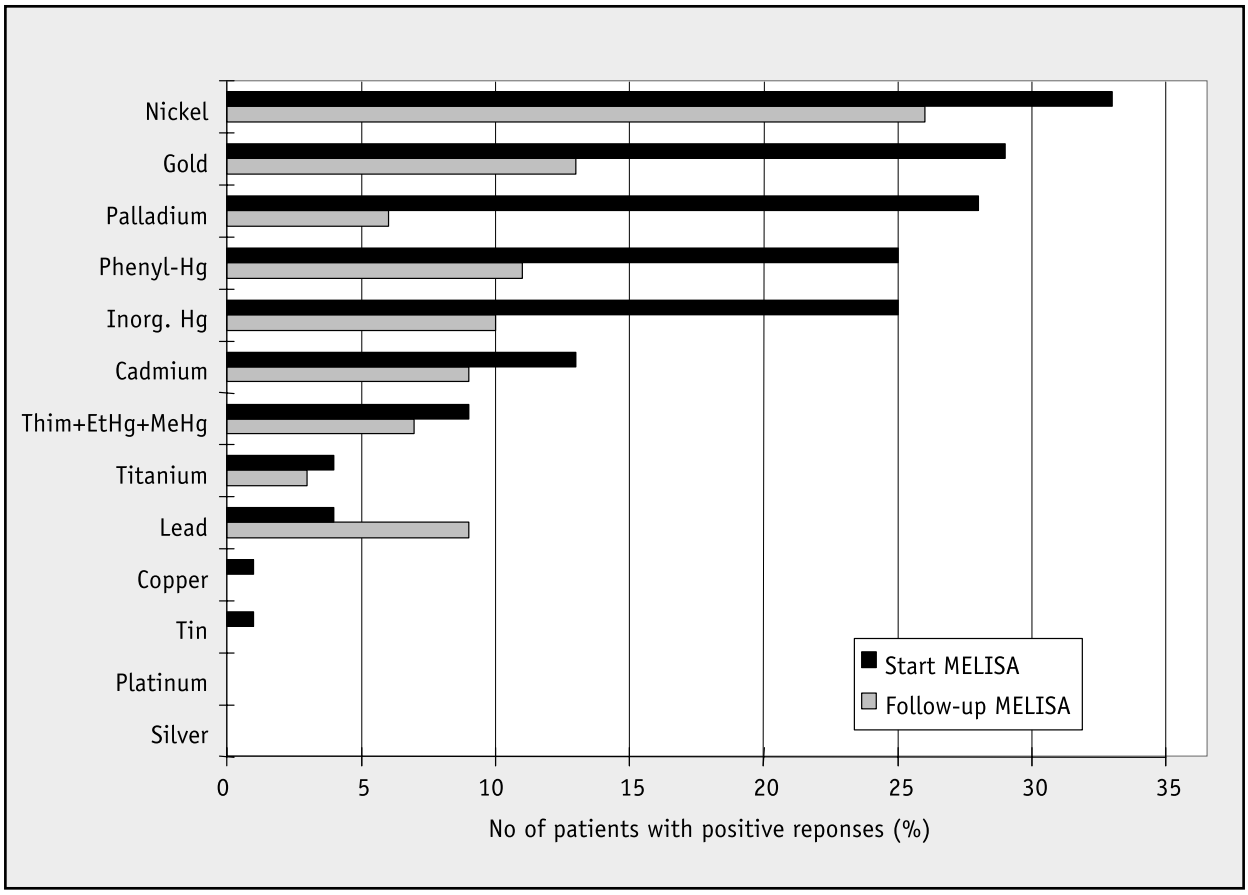

Fig. 6. Prevalence of positive responses to various metals in start- and follow-up MELISA®. MELISA is a registered trademark.

time that immunologic responsiveness to methylmercury has been studied in a larger population.

Man is exposed to methylmercury through consumption of contaminated fish and some reports indicate the possibility of formation of methylmercury from inorganic mercury by bacteria in the oral cavity and in the gastrointestinal tract [18]. The transformation of inorganic mercury to methylmercury by certain bacterial strains *in vitro* has been demonstrated previously [19, 20]. Since methylmercury is highly neurotoxic, the presence of methylmercury-specific lymphocytes in some patients indicates exposure and warrants further studies.

Phenylmercury salts are sometimes used as preservatives in eye drops and cosmetics and may induce allergic reactions [21]. In dentistry, phenylmercury has been used as a component of N2, a widely used root filling material [22]. The majority of patients had old root fillings, which may have contained N2. The use of phenylmercury in patch testing was discontinued in the last years despite the continued usage of phenylmercury. Only in a few cases, phenylmercury reactions occurred together with reactions to methylmercury or ethylmercury. Lymphocyte responses induced by two aliphatic mercurials, methylmercury and ethylmercury, partly cross-reacted [7], a fact confirmed recently by patch testing [23].

About 23% of the Uppsala patients with a CFS-like syndrome showed reactivity to inorganic mercury. These findings correspond well to the findings in 3,162 patients where the mercury-induced lymphocyte reactivity was about 20%. One could argue that mercury-induced lymphocyte responses might be mitogenic in nature. Some individuals could have a lower threshold for the triggering of lymphocytes by concentrations which are generally non-mitogenic. If this was the case, the removal of amalgam in sensitized individuals would have no impact on lymphocyte reactivity. The results of the follow-up study suggest that mercury-induced proliferation is truly allergenic in nature since the removal of mercury-containing amalgam resulted in reduced mercury-specific responses.

The finding of relatively high numbers of patients reacting to mercury compounds is supported by recent findings from Finland [24]. Laine and coworkers reported that 80 patients out of 118 with oral lichenoid changes displayed positive patch tests to dental metals. Seventy-six positive reactions were found to various mercury compounds but no positive reactions to acrylates were seen.

Metal intolerance is a growing problem as shown by the increasing number of patch test-positive subjects. Some of the sources of metal exposure include food and pharmaceutical additives, technical and cosmetic products, jewelry, and environmental pollution. The primary source of inorganic mercury exposure is dental amalgam as shown previously [25]. In an unselected Danish adult population (561 subjects), the prevalence of nickel-positive patch tests was 11% for women and 2% for men [26]. The frequency of reactions to Thimerosal ranked 3% in both sexes. The authors conclude that in the general population, IgE-mediated and cell-mediated hypersensitivity is common.

Compared to metal reactivity of patients with CFS, the responses in healthy subjects were significantly lower, thus confirming previously reported results [27]. A recent study has shown that nickel allergy, assessed by patch test, occurred in 36% of CFS patients in contrast to 19% in a control group [28].

We propose the following mechanisms behind multi-symptoms characterizing CFS: the binding of metals with strong affinity to SH-groups to autologous cell proteins may change their antigenicity and make such proteins foreign and therefore vulnerable to the attack of immunocompetent cells. Ongoing chronic inflammation and an increase in cyto-kines may affect the hypothalamic-pituitary-adrenal axis (HPA axis) and trigger nonspecific somatic and psychiatric symptoms [29]. The symptoms include profound fatigue, musculoskeletal pain, sleep disturbances, gastrointestinal and neurological problems, as well as other recognized features of chronic fatigue syndrome and fibromyalgia [29–32]. Following the removal of dental metals, the activity of the immune system is down-regulated and the patient improves. Japanese scientists have recently shown that a low metal diet and/or dental metal removal alleviated symptoms in 70% of patients with moderate to severe atopic dermatitis and positive patch tests to metals [33]. Following metal avoidance, systemic skin symptoms disappeared in the majority of patients. A similar protocol of allergen avoidance together with dental metal removal in sensitized patients is used for treatment of psoriasis in Kyoto, Japan [34] and in Neukirchen, Germany [35]. In the Kyoto Clinics, the sensitization to dental metals is diagnosed by the lymphocyte proliferation test [34].

A similar approach has also been used for the protection of workers occupationally exposed to beryllium. The factories involved in the production of beryllium-containing products routinely use the lymphocyte proliferation test for testing of beryllium-exposed individuals. The presence of beryllium-specific lymphocytes in the blood is taken as evidence of beryllium sensitization. Symptom-free workers exhibiting beryllium-specific lymphocyte proliferation are considered latently sensitized and at risk for development of beryllium disease. Such individuals are routinely relocated to a beryllium-free environment. About half of the beryllium-sensitized symptom-free subjects will develop beryllium disease if beryllium exposure continues [36].

The reason for increased susceptibility of certain individuals to metals or other chemicals could be due to inherited genetic polymorphism. Thus, in 365 patients with environmentally caused diseases, deficiencies in detoxification enzymes such as glutathione transferases M1 and glutathione-S-transferase 1 were found [37]. The subjects lacking the alleles for the above mentioned enzymes may be unable to detoxify xenobiotics. Under such conditions xenobiotics can be present in the organism for the time necessary for the induction of allergy or toxicity.

Conclusions

The results presented in this article indicate that lymphocyte sensitization to heavy and transition metals is common in patients with CFS-like syndrome and clinical metal sensitivity. Allergic reactions to metals are of delayed type hypersensitivity and usually involve the skin, oral mucosa, and the gastrointestinal tract. Since memory lymphocytes circulate through the body, they may, upon contact with tissue-bound metals, initiate an inflammatory process. Chronic inflammation may affect the HPA axis and trigger nonspecific symptoms characterizing CFS, fibromyalgia, multiple chemical sensitivity, and other multisymptomatic disorders. In a group of patients with CFS and metal hypersensitivity, removal of incompatible metal implants combined with anti-oxidant therapy resulted in down-regulation of metal-specific lymphocytes and long-term health improvement. The clinical relevance of *in vitro* findings is supported by *in vivo* worsening of systemic symptoms in connection with metal re-exposure such as dental treatment. Thus, the impact of metal implants on the general well-being of mankind might be greater than previously suspected. Finally, the study of the possible role of metal-specific lymphocytes in autoimmunity remains an exciting challenge for future studies.

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***Following laboratories perform routine MELISA® testing*:**

- Medilab, Stockholm, Sweden: www.medilab.se;
- Labor Schiwara, Bremen, Germany: www.medlab-schiwara.de
- Laboratoire Ategis, Rue des Chevaux 67, 1640 Rhode - St Genese, Belgium.

The test can also be obtained through:
- Institut für Umweltkrankheiten (IFU), im Kurpark 1, D-34308 Bad Emstal, Germany: www.ifu.org

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