

Metal-specific lymphocyte reactivity is down-regulated after dental metal replacement

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Submitted: January 18, 2006

Accepted: January 25, 2006

Key words: lymphocyte responses; allergy; dental restorations; amalgam, mercury; gold; nickel; metals; MELISA®

Neuroendocrinol Lett 2006; 27(1-2):101-000 PMID: ----- NEL271206A41 © Neuroendocrinology Letters www.nel.edu

Abstract

OBJECTIVES: This study was done to evaluate the results and clinical relevance of an optimized lymphocyte proliferation test, MELISA®, for metal-induced inflammation in patients with CFS-like symptoms. The treatment of patients consisted of the replacement of incompatible dental materials (RID) together with supportive anti-oxidant therapy.

DESIGN OF THE STUDY: 513 patients were tested by MELISA® at the beginning of the study. Out of this group, 248 patients were available for follow-up MELISA® after RID.

METHODS: In MELISA®, lymphocytes are isolated from the blood and cultivated with different metal salts in tissue culture medium containing 10% inactivated human AB⁺ serum or autologous serum. After 5 days, the presence of metal-reactive lymphocytes are measured by isotope labelling of newly formed DNA in growing lymphoblasts and evaluated by calculating the Stimulation Index.

RESULTS: Nickel was the most common sensitizer, followed by inorganic mercury, thimerosal, lead, cadmium, palladium and gold. After RID treatment, a decrease of metal-specific lymphocyte responses in patients who reacted to metals at the beginning of the study could be observed. The cultivation of lymphocytes in autologous and homologous serum did not significantly affect the results. Simultaneous, the health status of patients improved as well.

CONCLUSIONS: Replacement of incompatible dental materials resulted in down-regulation of metal-induced lymphocyte sensitivity *in vitro*, as well as in the improvement of health status of majority of patients with unspecific CFS-like symptoms.

Abbreviations:

- cpm – Counts per minute
- CFS – Chronic Fatigue Syndrome
- Hg – Mercury
- LST/LTT – Lymphocyte Stimulation Test / Lymphocyte Transformation Test
- MELISA® – Memory Lymphocyte Immuno Stimulation Assay
- No. – Number of patients
- ns – Non significant
- P – Value of probability for statistical significance
- RID – Removal of Incompatible Dental Materials
- RPMI 1640 – Roswell Park Memorial Institute (culture medium)
- SH groups – Sulphydryl groups
- SI – Stimulation Index

Introduction

Dental amalgam, which is composed of at least 50% metallic mercury together with silver, copper, tin, palladium and zinc, has been used by dentists to restore decayed teeth for over 150 years. When amalgam interacts with saliva, it will slowly corrode [7, 8]. Dental amalgam fillings were thought to be safe *in vivo* based on the erroneous belief that mercury, once bound to other metals, became an inert compound. It is now firmly established that mercury is continuously released into the oral cavity and consequently inhaled, absorbed and ingested [18, 16]. With the help of radioactive ²⁰³Hg it has been shown that mercury is deposited in many organs, including the brain [13, 14, 11, 12, 10, 33]. Mercury and other heavy and transition metals, such as gold or silver, strongly bind to SH groups in autologous proteins and therefore have potential to elicit allergic and autoimmune reactions. Inorganic mercury and gold induce autoimmunity in genetically susceptible rats and mice, and susceptibility or resistance to autoimmunity is dependent on genetic haplotypes [3].

Metals may also induce allergy in genetically susceptible individuals [19, 20, 22]. Allergic reactions are mostly of type 4 (delayed-type hypersensitivity, such as contact dermatitis) but immediate-type reactions are sometimes also observed [2, 17]. Cell-mediated reactions operate in parts of the body different from those where metals are deposited [12, 28].

Metal-induced hypersensitivity in man is based upon the reaction of the allergen with the surface of memory T-lymphocytes previously sensitized to a specific allergen. Upon contact with the allergen, memory cells become activated and produce lymphokines. The interaction of memory cells with the antigen forms the basis of the Lymphocyte Stimulation Test (LST), also called Lymphocyte Transformation Test (LTT). MELISA® (MEemory Lymphocyte Immuno Stimulation Assay) is an optimization of LST. With MELISA®, it is possible to measure immunological sensitization induced by mercury, gold, palladium, and other metals included in dental amalgam and in other metal alloys [25, 26, 27]. Additional sources of metals are metal pigments such as titanium dioxide

(TiO₂, E171), a white colouring agent used e.g. in composites, dental cements, and different pharmaceuticals [27]. Phenylmercury has been used together with lead and arsenic in certain root fillings [6]. Thimerosal, an organic mercury preservative, was used in nose and eye drops, vaccines, and in cleaning solutions for soft contact lenses in the past and is still used as a preservative in certain vaccines. Nickel was also tested, as it is the most common sensitizer among the metals.

In this study we performed the MELISA® test on patients with health problems suspected to be related to amalgam and other dental metal alloys. Lymphocyte reactivity was studied prior to and after the replacement of biological incompatible dental restorations. In addition, lymphocytes were cultured in homologous or in autologous serum (patient's own serum) in order to see if the latter might contain factors which might change the reactivity of lymphocytes. It was found that replacement of incompatible dental materials down-regulated metal-specific responses in sensitized individuals.

Material and methods

Patients

Patients from the County of Uppsala were referred to the Department of Clinical Metal Biology at The University Hospital, Uppsala, Sweden, by physicians and dentists, while patients from other parts of Sweden were referred by physicians only. The Clinical Metal Biology Department has been established by Uppsala Health Community in 1991 with the aim to develop a better way of taking care of patients with unspecific symptoms and possible metal etiology. The patients underwent comprehensive medical examination and differential diagnosis including routine laboratory tests and special tests, among others an *in vitro*-test for metal allergy (MELISA®). The clinical metal allergy often reported by patients and the worsening of symptoms following dental treatment, raised suspicion of adverse effects from dental materials. The characteristics of the patients are shown in Table I.

Most patients suffered from chronic or long-persisting ill health. The medical problems affected many parts of the body expressed as ambulatory muscular-skeletal pains, disturbances in the function of the gastro-intestinal system, hormonal perturbations as well as psychological symptoms. The key symptom was profound chronic

Table I. Characteristics of patients.

	Females	Males
Nr. = 248	188 (76%)	60 (24%)
Range (yr)	18–75	17–65
Age, mean	44,4	44,8
Age, median	44,5	44

fatigue. Detailed description of the symptoms and their prevalence was described previously by Lindh et al [15].

Most patients had metallic dental restorative materials such as amalgam, gold alloys and metallic posts. They also had non-metallic restorations such as composites and ceramic crowns and bridges. Additional exposure was observed emanating from environmental allergens such as those present in cigarette smoke, metals from earrings or other jewellery, organic mercury (such as ethylmercury from vaccines and methylmercury from fish), chemicals and mould. From the anamnesis, it was observed that a majority of patients had experienced aggravation of symptoms 2–7 days after dental treatment. Most of the patients reported clinical metal hypersensitivity such as skin problems from contact with metallic jewellery and jeans buttons.

When the informed patient agreed, the treatment consisted of replacement of amalgams and other restorative materials containing metals to which lymphocytes were reacting on an individual basis. Patients were referred to dentists aware of the fact that dental restorations might cause allergic reactions in susceptible patients and who therefore took special care in minimizing the metal exposure during the replacement procedure [15]. Regular meetings with doctors, nurses, dentists and dental technical personnel were held in order to increase the knowledge in the group working with these patients. All patients underwent antioxidant treatment and when indicated, immunosuppressive therapy with steroids was used to counteract side-effects during the dental treatment; as described in detail by Lindh et al [15].

MELISA[®]

Blood samples for MELISA[®] testing were taken from patients during the first medical investigation (before treatment) and after treatment (follow-up). The follow-up period varied from one to four years but was two to three years for the majority of patients. Some of the patients were followed for four years. Reactivity of lymphocytes to metals was assessed by the uptake of tritiated thymidine as described previously [25, 26, 27]. 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 10⁶ cells per ml. RPMI 1640 medium was enriched with 10% heat-inactivated normal human AB⁺ serum (homologous serum), or with patient's own serum (autologous serum), gentamicin and L-glutamine. The cells were cultured in 48-well tissue culture plates, coated with different concentrations of metal salts. Two-fold dilutions were used for each metal salt, and in duplicates, per patient. Three wells with 100 µl of water but without metal solutions were used as negative controls and provided information about the spontaneous proliferation of the lymphocytes. Purified Protein Derivative (tuberculin, Statens Serum Institut, Denmark) was used as a positive control for estimation of the normal function of cell-mediated immunity. After 5 days incubation at 37°C, an aliquot of cells from each well was transferred to a

new plate and supplemented with methyl-³H-thymidine (Amersham, England; spec. act. ~3.2 TBq/mmol) per well. After 4-hour incubation, cells were harvested in an automatic cell-harvester (Inotech, Switzerland) and the radioactivity was measured using liquid-scintillation counter (LKB/Wallack, Finland). The increase in ³H-thymidine incorporation in antigen-treated cultures was expressed as Stimulation Index (SI), which, is defined as the intensity of lymphocyte responses to metals; it is calculated as the isotope uptake in antigen-treated cultures divided by the mean isotope uptake in untreated control cultures. Lymphocyte responses induced by metals were characterized by the mean of the two highest responses obtained with each metal salt. We consider SI equal to or more than 3 as positive response.

For histology evaluation, 100 µl of cell suspension was taken from 5 days positive cultures and smears were prepared in a cytocentrifuge (Shandon, England) and stained with May-Grünwald/Giemsa [28]. The results were considered positive only if increased incorporation of ³H-thymidine was confirmed with the presence of lymphoblasts on cell smears.

MELISA[®] is a biological test where the lymphocytes are dependent on a blood-like surrounding. In the original description, 10% human heat-inactivated AB⁺ serum was used to give a standardized test for metal reactivity. New sera were tested for suitability to support metal-specific lymphocyte proliferation *in vitro*, using lymphocytes from donors with known metal-allergy. Only blood donors with a background of 1000–4000 cpm were accepted and hence their sera were pooled to a serum batch of around 3 liter. Autologous serum, taken at the same time as blood samples for MELISA[®] testing, was prepared by clotting and centrifugation and heat inactivated in the water bath at 56 °C for 1 hr.

Statistical analysis of data was performed using the Wilcoxon signed ranks test, as well as the chi-2 test for the difference between groups.

Patients with spontaneous blastogenesis i.e. who have their negative controls more than 10,000 count per minute (cpm) were excluded from the study since the high background value affects the sensitivity of the test (unpublished).

Criteria for estimation of changes in the positive lymphocyte responses after treatment:

In an attempt to determine the percentage of patients who showed changes in their mean Stimulation Index after treatment, the changes in lymphocyte responses were categorized as follows:

- 1) SI value decrease of 30% or more from prior treatment is considered a significant decrease,
- 2) SI value increase of 30% or more from prior treatment is considered a significant increase, and
- 3) SI value decrease or increase of less than 30% from prior treatment is considered as no change.

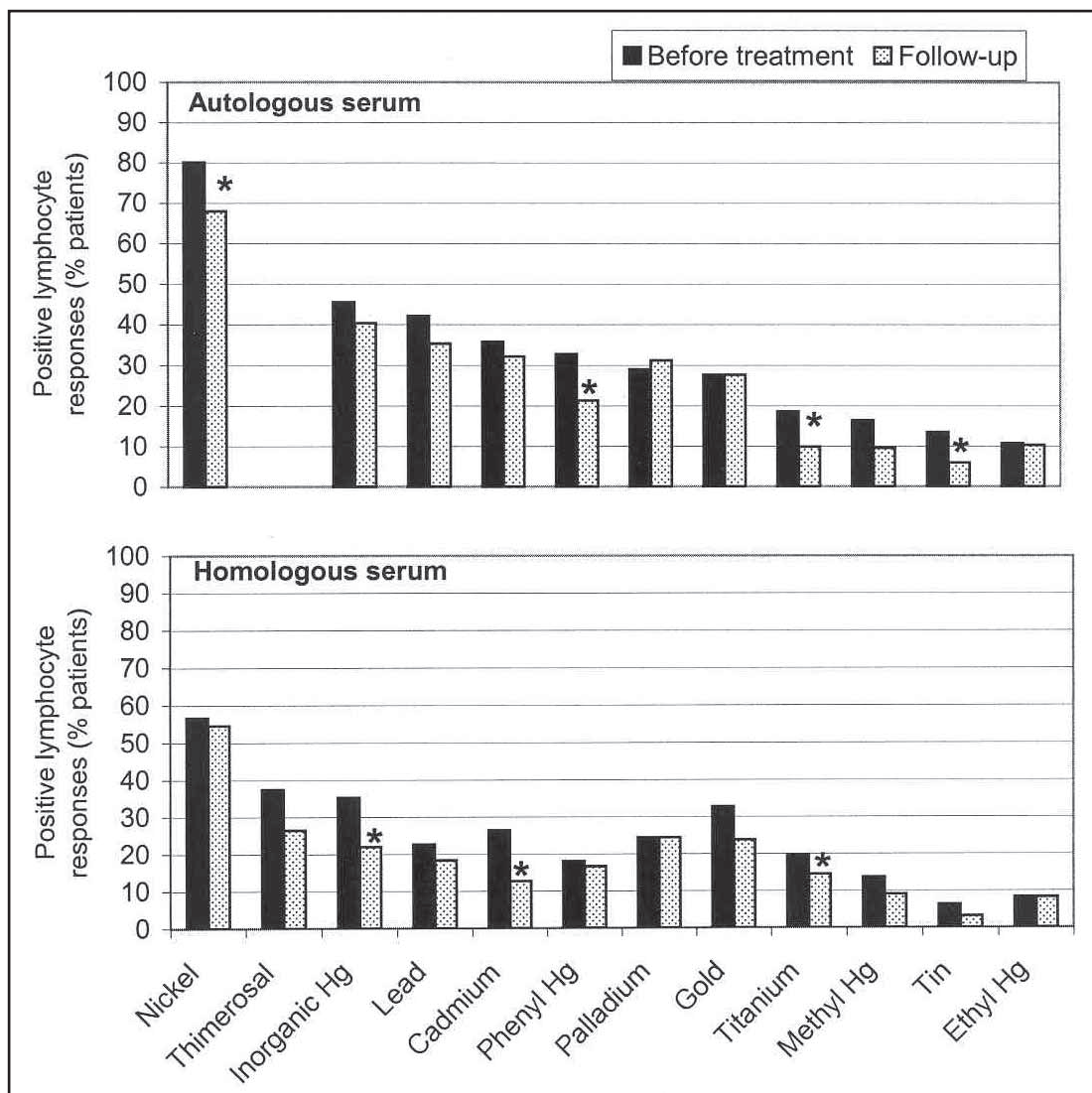


Figure 1. Prevalence of metal sensitivity in patients before treatment and follow-up MELISA®. Test performed with autologous serum (nr. 141) and homologous serum (nr. 107)
 *= significantly different ($p < 0.05$).

Results

The prevalence of positive responses to metals in the MELISA® test, performed with autologous or homologous serum is shown in Figure 1. In the autologous serum group the prevalence is higher than that in the homologous serum group. Nickel was the most common sensitizer in both groups (80% and 56% autologous and homologous, respectively) followed by inorganic mercury, lead, cadmium, phenylmercury, palladium, gold, titanium, methylmercury, tin and ethylmercury in the autologous group. In the homologous group, where thimerosal was included, it comes after nickel in frequency followed by inorganic mercury, gold, cadmium, palladium, lead, titanium, phenyl-, methyl-, and ethyl mercury and tin.

Mean Stimulation Indexes prior to RID treatment and in follow-up MELISA®, are shown in Figure 2A and

2B. After treatment, lymphocyte responses decreased significantly to all metals in the autologous serum group (Figure 2A). In the homologous serum group, there was a significant decrease in the response to most of the metals, except for thimerosal, methylmercury and tin, where the decrease was not significant (Figure 2B).

The changes in mean Stimulation Indexes for various metals in patients are shown in Figure 3A and 3B. Following RID treatment, a 75% decrease of lymphocyte reactivity was obtained in the autologous serum group (Figure 3A) and 82% in the homologous group (Figure 3B). Twelve percent and 9% (autologous and homologous, respectively) of the patients showed no change in their lymphocyte reactivity and 13% of autologous group and 9% of homologous group, showed an increase in SI.

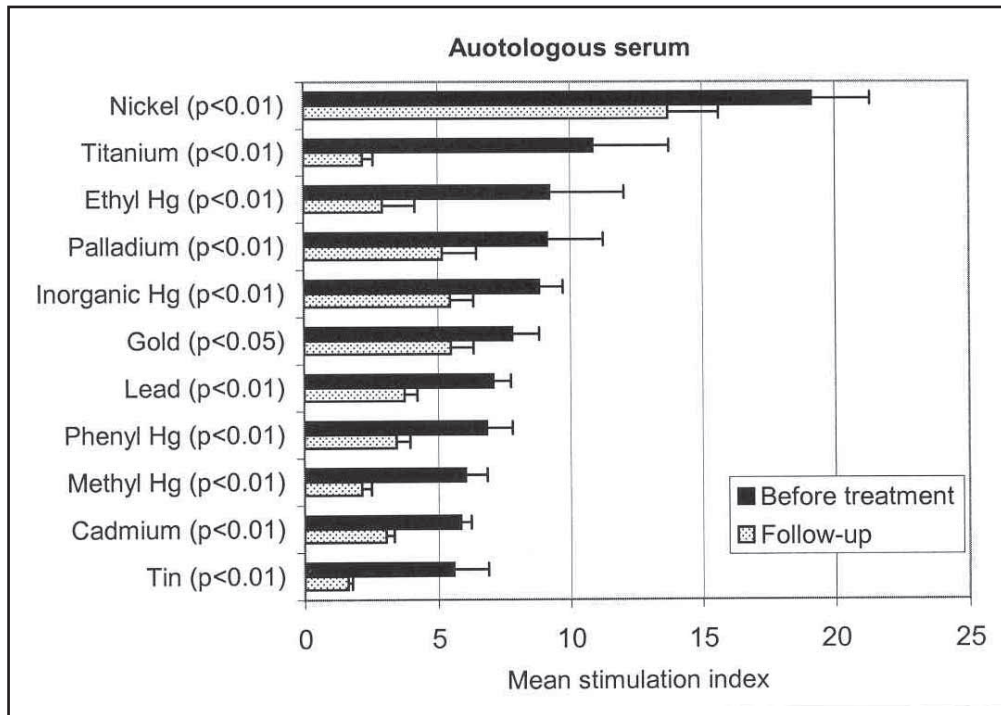


Figure 2A. Mean positive responses of patients before treatment and follow-up MELISA® (nr. 141, autologous serum). The numbers in brackets show the level of significance (bars are mean ± SE).

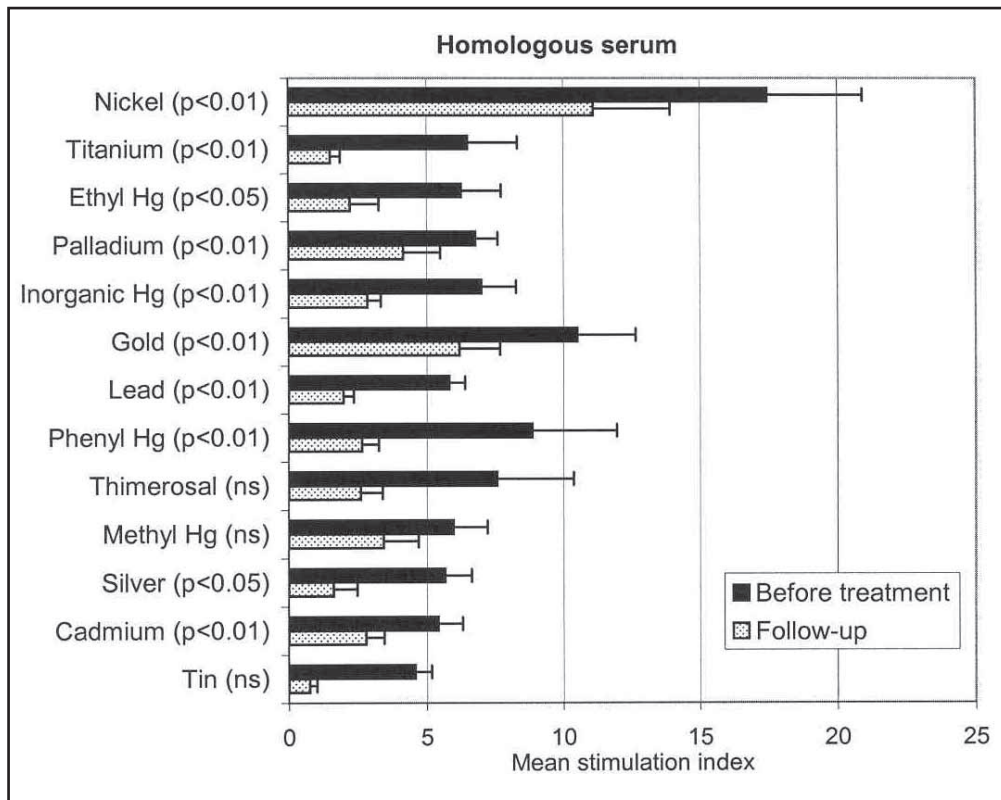


Figure 2B. Mean positive responses of patients before treatment and follow-up MELISA® (nr. 107, homologous serum). The numbers in brackets show the level of significance (bars are mean ± SE).

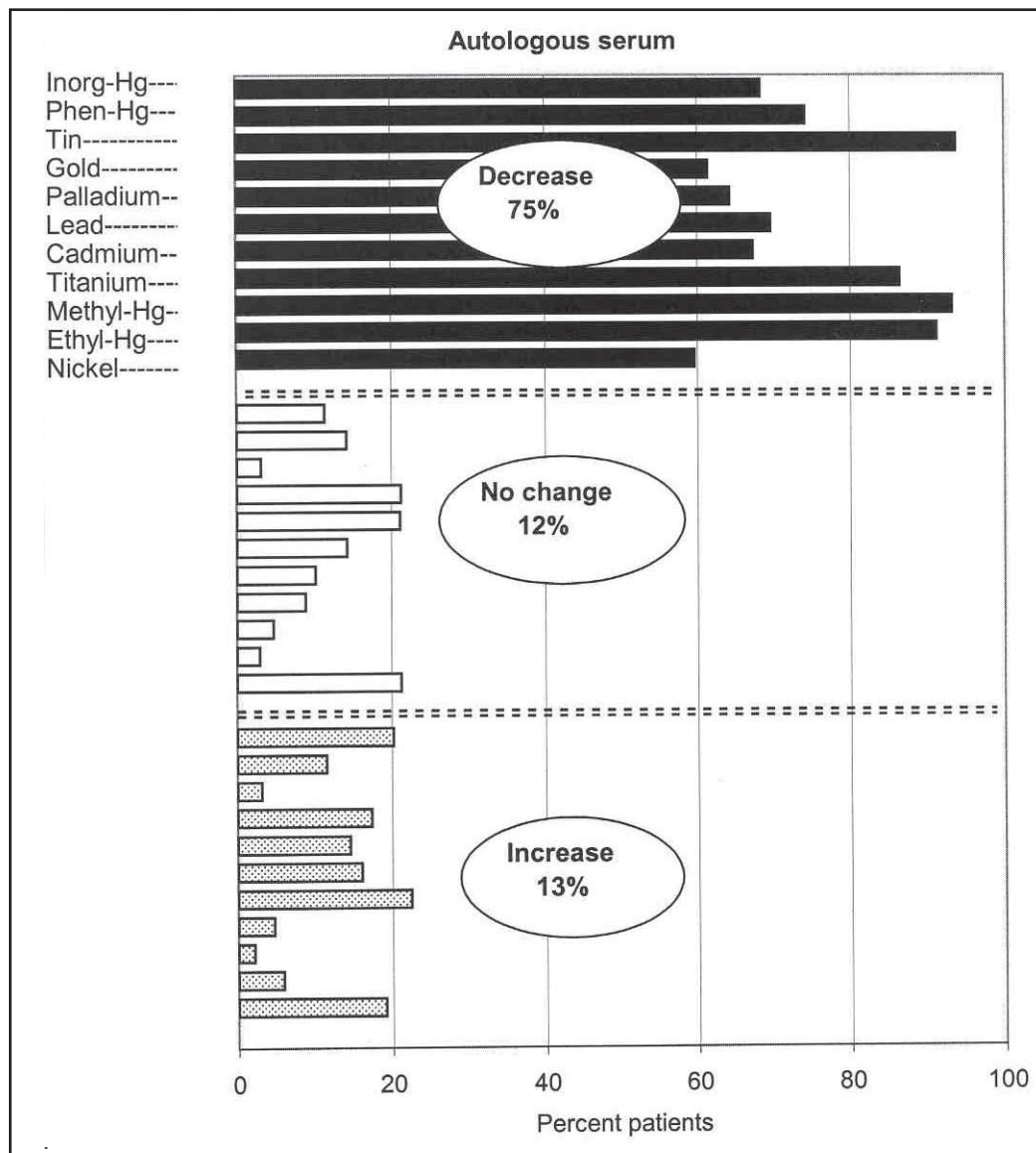


Figure 3A. Changes in lymphocytes responses (mean stimulation index), after treatment as compared to state prior treatment. Black, empty and dotted bars correspond to percent of patients with decreased, unchanged and increased responses, respectively. The same order of metals in black bars is also valid for the empty and dotted bars (nr. 141, autologous serum).

Discussion

A significant number of patients with CFS-like symptoms had metal-specific lymphocytes in the blood. Nickel was the most frequent sensitizer, followed by inorganic mercury, thimerosal, and other metals. The high prevalence of nickel hypersensitivity in some patients was due to wearing earrings and other jewellery which contained nickel. About two thirds of the patients studied were females [28]. Inorganic mercury is the second most common sensitizer which implies the role of mercury from dental amalgam as a possible causative agent for different health problems related to amalgam. According to Svare [30] and WHO criteria [34], amalgam is the main source of inorganic mercury exposure in man.

The main exposure paths for mercury from amalgam fillings are absorption by the lungs from intra-oral air; vapor absorbed by saliva or swallowed; amalgam particles which may be ingested, and membrane, olfactory, venous and neural path transfer of mercury absorbed by oral mucosa, gums, etc [1, 32, 4, 9]. The response of lymphocytes to thimerosal was very much expected since thimerosal was previously used in Sweden and worldwide as a preservative in vaccines, soft contact-lens fluids and immunoglobulin preparations. Sensitization to gold, palladium, and titanium was also expected from the frequent use of different metal alloys in dental restorative materials. Sensitization to phenylmercury may be due to

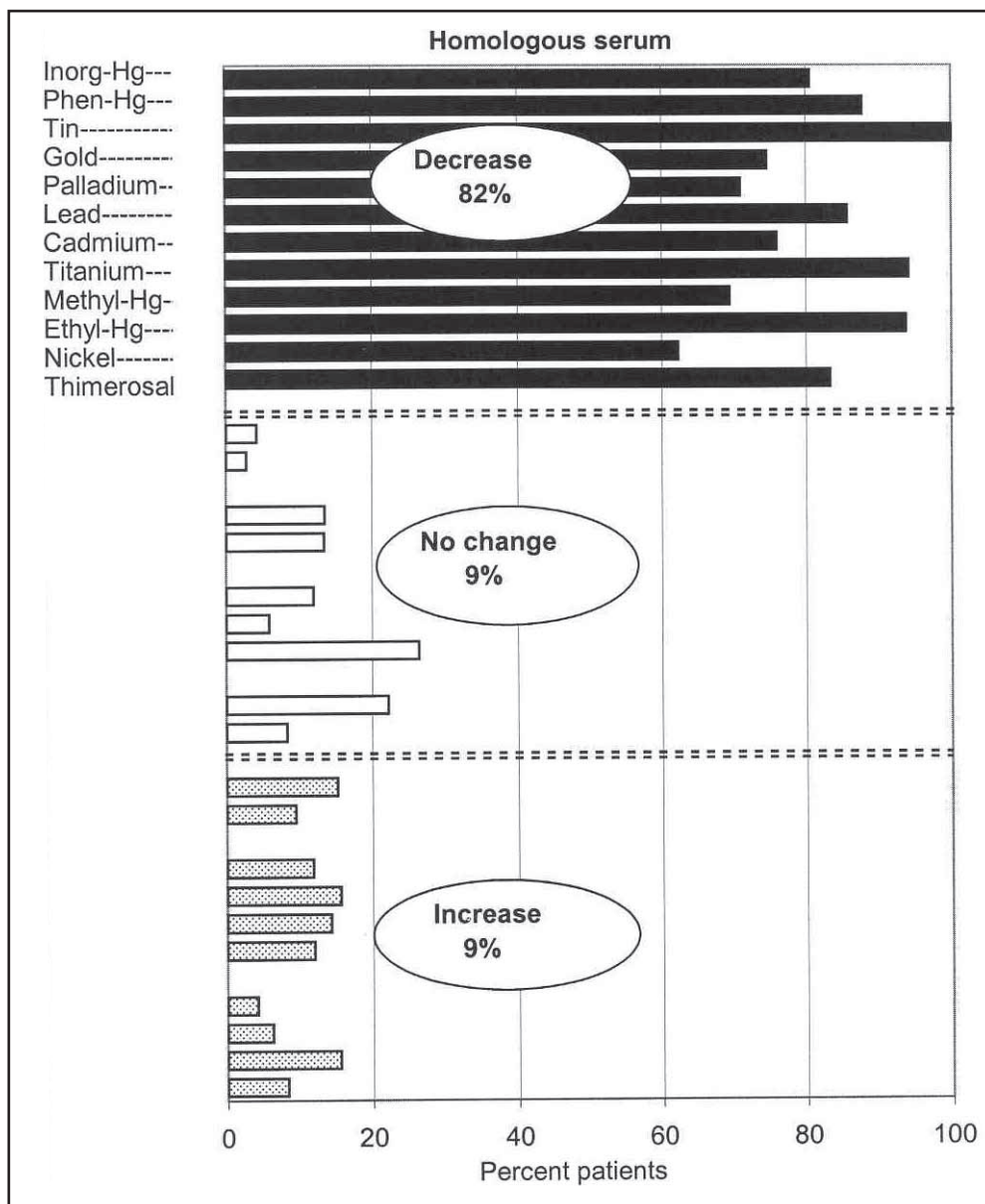


Figure 3B. Changes in lymphocytes responses (mean stimulation index), after treatment as compared to state prior treatment. Black, empty and dotted bars correspond to percent of patients with decreased, unchanged and increased responses, respectively. The same order of metals as in black bars is also valid for the empty and dotted bars (nr. 107, homologous serum).

use of phenylmercury salts as preservatives in eye drops and cosmetics. Phenylmercury may induce allergic reactions [16]. In dentistry, phenylmercury has been used as a component of N2, a widely used root canal filling material [6]. Between 10–20% of the patients have been previously reported to respond to methylmercury [28], and this have been confirmed by our findings. The main source of methylmercury is fish and Swedish fish meal is also used as a source of protein in feed for poultry and swine [24], in addition to fish, meat and eggs from animals fed on fish products are likely to contribute to the exposure to methylmercury in the general population.

After replacement of dental metals in patients, there was a general decrease in patients' responses to metals in the follow-up MELISA® test. A significant decrease in positive responses to inorganic mercury in patients after amalgam replacement indicates that this lymphocyte reactivity is allergic in nature. These results corroborate the data previously published [28].

Regarding methodology, MELISA® is one of the two few validated LTT tests [31], the other being beryllium-specific LTT used in US as a golden standard for the evaluation of beryllium allergy in occupationally exposed workers [23].

The use of patient's own serum (autologous) has theoretically the advantage that it might better reflect the actual situation of accelerators and inhibitors of lymphocyte metal reactivity in the individual patient compared with homologous serum. During evaluation of the results, no significant differences have been noticed in lymphocytes reactivity regarding the use of homologous or autologous serum in the MELISA® test.

In addition to the replacement of incompatible dental metals, the patients were informed about their existing metal sensitivity and avoided future exposure to metals as a precaution. The efficacy of a low nickel diet for the treatment of CFS has been published previously [21]. A similar reduction in metal exposure can be achieved by quitting smoking or avoiding cigarette smoke. It has been reported that cigarette smoke contains manganese, mercury, cadmium and lead [5]. In recent publications, the prognosis of improvement of multiple sclerosis and other autoimmune diseases following amalgam removal has been inversely correlated to the exposure to nickel and other metals from cigarette use [2].

Thus for patients who are genetically susceptible to metal pathology, the strict avoidance of metal exposure is strongly recommended.

Conclusions

The replacement of incompatible dental restorations in sensitized patients together with anti-oxidant supplementation resulted in the down-regulation of metal-induced lymphocyte responses *in vitro*. The reduced ability of lymphocytes to respond to metals *in vitro* after dental metal replacement might reflect the decrease of chronic inflammation *in vivo*. Thus, *in vitro* testing is not only improving diagnosis but is also useful for further monitoring of the outcome of the treatment.

Acknowledgments

The authors would like to thank the personnel at the former Department of Clinical Metal Biology, University Hospital, Uppsala. Financial support from the Ministry of Health and Social Affairs as well as the National Board of Health and Welfare is duly recognised.

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