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Detection of sensitization to low molecular weight chemicals

Inventory and evaluation of *ex vivo* methods

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Advisering en kennisbasis sensibilisatie

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1 Background

Chemical-induced allergic diseases can be evoked after exposure to low molecular weight (LMW) chemicals. These chemicals can induce allergic contact dermatitis after skin exposure or respiratory allergies (i.e. allergic rhinitis, rhinoconjunctivitis and asthma) after inhalation exposure. Allergic contact dermatitis is a T cell mediated disease, classified as a type IV or delayed type hypersensitivity reaction. Contact sensitizers are in general LMW compounds that can only induce sensitization when they are capable of penetrating the skin and binding to proteins in the epidermis. After binding to proteins, hapten-carrier complexes are formed that can be recognized by cells of the immune system (Kimber et al., 2002). Exposure occurs at the workplace or at home, since many consumer products (cosmetics, clothing, toys, and jewellery) contain contact sensitizers (Wijnhoven et al., 2008). Chemical-induced respiratory allergy is not as common as contact dermatitis, but the symptoms are severe and sometimes even fatal, i.e. rhinitis, wheeze, and asthma (Boverhof et al., 2008). The disease can be induced by low and high molecular weight chemicals. LMW chemicals can induce either type I hypersensitivity responses (immediate, IgE-mediated) leading to allergic asthma or type IV hypersensitivity responses, leading to extrinsic allergic alveolitis or hypersensitivity pneumonitis (Garssen et al., 1991; Sastre et al., 2003; Mapp et al., 2005). These allergic diseases develop in two phases: an asymptomatic *sensitization* phase, in which the immune system is primed and an *elicitation* phase, in which after subsequent exposure the symptoms become manifest.

Chemical contact and respiratory allergies are important occupational problems and preventive strategies that reduce this burden are therefore important. These strategies are, i) primary prevention: reduction of the exposure to the chemical, ii) secondary prevention: detection of the disease in an asymptomatic phase, and iii) tertiary prevention: improve the health state of subjects that have developed an allergy. For secondary prevention it is important to identify people that are sensitized in an early stage, since it has been shown that the prognosis in these subjects can be improved, for instance by protecting them from further exposure to the chemical. At workplaces where exposure to sensitizers occurs, periodically screening for sensitization is therefore an important tool to detect the development of chemical-induced allergy in an early stage (Gezondheidsraad, 2008). In addition, to reduce the burden of chemical-induced allergies in the general population, many legal frameworks exist. Consumer products that contain sensitizing chemicals should be labelled accordingly. Furthermore, for certain contact sensitizers concentration limits are set, for example for nickel and preservatives in order to prevent sensitization. In addition, some sensitizing agents have been banned from the market and replaced by other chemicals, for instance the preservative methyl dibromoglutaronitrile. To assess the efficacy of these legislative measures it is important to assess what the effects are on the prevalence of chemical-induced allergies. Current knowledge shows that consumers are predominantly exposed to contact sensitizers and not to respiratory sensitizers and most of the epidemiological surveys have therefore been monitoring trends in contact dermatitis, by using the diagnostic patch test.

The patch test is used as a diagnostic tool for over a century and is considered to be the 'gold standard'. Standardized patch test systems are commercially available (i.e. the European Standard Series and the True Test). The principle of the test is that the chemicals are applied in an aqueous solution on the back of patient and stay there for two days under occlusion. Skin reactions are then read by a dermatologist on the day of removal, and 1 and 2 days after removal. The severity of the patch test is subjectively graded in weak, strong and extreme positive reactions (Belsito, 1997). For respiratory allergies, different diagnostic tools can be used, dependent on the type of immune reaction that is induced. For substances that induce IgE, skin prick testing can be used. For this test, the skin is pricked with a needle containing a small amount of the allergen. In sensitized persons, a wheal develops, indicating the

presence of antigen-specific IgE. In addition, specific IgE can be measured in serum. However, not all LMW chemicals induce IgE, and especially for di-isocyanates this method will not be a reliable tool for the detection of sensitized persons (Mapp et al, 2005). It is possible to use the patch test to measure sensitization to respiratory sensitizers as well.

Although the patch test is used for a long time as a diagnostic tool, it is questionable if this test is the most appropriate one for periodically screening of sensitization in the workplace or for assessment of the prevalence of contact dermatitis in the general population. One of the most important disadvantages is the invasive nature of the test. Concerns exist that the patch test can induce active sensitization, which has been shown for fragrances (White et al, 2008) and *para*-phenylenediamine (PPD) (Devos & Van der Valk, 2001), but in general appears to be a rare event (Jensen et al., 2006, Dawe et al., 2004). Also, in a population-based study it has been shown that PPD did not induce active sensitization (Thyssen et al., 2007). Furthermore, correct interpretation of the patch test can only be done by an experienced dermatologist (Bruze et al., 1995). Like many test methods, the patch test can give false-positive results, for example due to irritant reactions. False-negative test results may occur when low concentrations of a substance are used, due to local corticosteroid therapy or after sun exposure (Mowad, 2006, Traidl-Hoffmann & Ring, 2008). In a preclinical stage, i.e. for the periodically screening for sensitization, a less invasive test would be preferable.

A possible alternative test for immune responsiveness to LMW chemicals may be the lymphocyte transformation test (LTT), which is less invasive than the patch test. The LTT is based on the principle that during the sensitization phase, T lymphocytes become activated and develop into effector and memory T cells. These memory T cells recognize the antigen during a second encounter. This can also be mimicked *in vitro* by using lymphocytes from patients and culture them with the suspected contact allergen. In patients sensitized to this chemical, T lymphocytes are activated and start proliferating and excreting soluble mediators, such as cytokines and chemokines. The LTT is performed in peripheral blood mononuclear cells (PBMCs) and in most studies not only PBMCs from patients are used but control subjects without contact dermatitis are included as well. The cells are cultured with a range of non-toxic concentrations of the contact allergen for several days. Proliferation is assessed by measuring the incorporation of a radioactive marker, for instance [³H]-Thymidine. Besides the LTT, a commercially available method, the MELISA test (memory lymphocyte immunostimulating assay), might be a potential test for the detection of allergy induced by LMW chemicals. This test is based on the same principle as the LTT but uses a slightly modified protocol. The differences are that before culturing the PBMCs with the suspected chemical, the monocyte content is reduced, which should reduce the number of false-positive results. Furthermore, after five days of culturing not only cell proliferation is assessed, but the cultured cells are morphologically examined for the presence of lymphoblast to confirm the proliferation data (Stejskal et al., 1994).

An *ex vivo* method for the detection of sensitization to LMW chemicals would be a useful tool in periodically screening for sensitization at the workplace. Furthermore, since such a test is less invasive than the patch test, it could be useful in epidemiological surveys that study trends in the prevalence of contact dermatitis. Therefore, a literature survey was conducted to make an inventory and evaluation of potential *ex vivo* methods for the assessment of sensitization by LMW chemicals.

2 Methods

A literature survey was conducted using the following sources:

All publications that are listed on the website www.ivpt.info has been used. This website provides an overview of the published *in vitro* patch tests

Additional relevant literature was searched using PubMed and Scopus electronic databases.

All *ex vivo* methods are summarized in Appendices 1 and 2 and if possible the sensitivity and specificity of the assays was calculated. The *sensitivity* of an assay is the proportion of actual positive subjects which are correctly identified as such. The *specificity* of an assay is the proportion of negative subjects which are correctly identified. The number of false-positives, i.e. the people without contact dermatitis which have a positive test and the number of false-negatives, i.e. the number of people with contact dermatitis that have a negative test, are important parameters that can be used to assess the accuracy of a method.

3 *Ex vivo* methods for the detection of sensitization to LMW chemicals

3.1 LTT: proliferation

The majority of studies have used the LTT to detect nickel allergy. The assays differ in sensitivity, ranging from 58% - 100% (Macleod et al., 1970; Svejgaard et al., 1978; Silvennoinen-Kassinen, 1981; Kimber et al., 1990; Rasanen & Tuomi, 1992; Cederbrant et al., 1997; Lisby et al., 1999; Falsafi-Amin et al., 2000; Cederbrant et al., 2003; Spiewak et al., 2007). The accuracy of a test is determined not only by its sensitivity but also by its specificity. One of the tests that had a sensitivity of 100% had a specificity of 0%, hence, all controls were false-positive (Cederbrant et al., 1997). The specificity of the other LTT that was 100% sensitive was better; it was 80% (Kimber et al., 1990).

Besides nickel other metals have been tested in the LTT. Chromium was tested in several studies. The sensitivity in most studies was moderate (62.5-75%) and the specificity was moderate to good (75-100%) (Christiansen et al., 1980; Al-Tawil et al., 1983; Rasanen et al., 1991; Lindemann et al., 2008; Martins et al., 2008). The LTT appears not to be able to detect cobalt allergy, the sensitivity was only 43% (Veien & Svejgaard, 1978). The specificity of the LTT for gold and palladium was very low, almost half of the controls were positive (Cederbrant et al., 1997).

In order to improve the LTT, adjustments have been made in the protocol. One of these is the addition of cytokine cocktails during the culture period (Rustemeyer et al., 2004; Moed et al., 2005; Spiewak et al., 2007). The cytokine cocktails contained either a combination of IL-7 and IL-12 (Th1 cocktail) or IL-7 and IL-4 (Th2 cocktail). IL-7 plays an important role in the enhancement of cell proliferation, IL-4 is involved in the differentiation of naïve T lymphocytes into Th2 cells, and IL-12 is involved in the differentiation of naïve T lymphocytes into Th1 cells. It has been shown that in contact dermatitis both Th1 as Th2 lymphocytes play a role (Probst et al., 1995, Minang et al., 2005).

Rustemeyer et al. (2004) have shown that the addition of a Th2 cytokine cocktail increased the sensitivity of the LTT for nickel from 63% to 91%. Addition of Th1 cytokines had fewer effects on sensitivity, an increase to 77% was observed. Furthermore the specificity decreased when Th1 cytokines were added from 75% to 64%. In this study it was shown that the sensitivity of all LTT assays was higher than the sensitivity of the patch test, which was only 54%. Moed et al. (2005) used the modified LTT to assess allergies for nickel, chromium, cobalt, fragrance mix, PPD and methyl-chloro-isothiazolinone (MCI). In this study it was shown that the addition of Th2 cytokines did not increase sensitivity of the LTT for nickel but decreased both sensitivity and specificity. For the other contact sensitizers, the addition of cytokine cocktails improved the sensitivity of the LTT. However, for cobalt, chromium, fragrance mix and MCI the sensitivity was still very low. For PPD addition of Th1 cytokines greatly improved the sensitivity of the LTT from 17% to 71% (Moed et al., 2005). Spiewak et al. (2007) have refined the LTT for nickel by using the same cytokine cocktails. In this study it was shown that the addition of Th2 cytokines increased the correlation with the patch test from 0.61 to 0.74.

Besides metals, other contact sensitizers have been tested in the LTT, but to a limited extent. The LTT could not be used to identify patients with a contact allergy for isothiazoline, the sensitivity was only 50% (Stejskal et al., 1990). For thiuram allergy, the LTT might be a sensitive alternative, since the

accuracy of this test was 100%. It is important to note that this study was performed in a small study population and should be repeated in a larger group (Kimber et al., 1991). Besides the study of Moed et al. (2005) two other studies used the LTT, but in one study no control group was included, so it is impossible to evaluate the accuracy of this method (Skazik et al., 2008). In the study of Sieben et al. (2002) it was shown that the LTT could be used to identify contact allergy for PPD. To obtain a sensitivity of 100% it was necessary to incubate the cells with Bandrowski's base, the active metabolite of PPD. The metabolic capacity of this *in vitro* system is possibly not sufficient to metabolize PPD.

The applicability of the LTT to assess fragrance allergy has been tested in one study. The sensitivity of the LTT was low when the LTT was performed with all chemicals of fragrance mix I. The individual fragrances were tested as well, and the sensitivity of the LTT for oak moss and isoeugenol was good. For the other fragrances the sensitivity was very low. The specificity of this test cannot be determined, since there was no control group included (Sieben et al., 2001).

3.2 MELISA

The commercially available MELISA test has been developed as a blood test for the detection of metal allergy. Cederbrandt et al. (1997) compared the MELISA and the LTT to detect allergy for nickel, gold and palladium. The sensitivity for nickel was slightly higher in the MELISA than the LTT, but the specificity was low in both assays. For gold the LTT was more sensitive, but less specific than the MELISA. For the detection of palladium allergy, the MELISA was the most sensitive assay, but the LTT gave a good sensitivity as well. However, the specificity for palladium was low in both assays (Cederbrandt et al., 1997). The MELISA test could identify all nickel allergic patients correctly in another study. However, in this study it was also shown that the specificity for nickel was low (Valentin-Thon et al, 2006).

3.3 LTT: cytokines

The measurement of proliferation in the LTT sometimes fails to distinguish between patients and controls. Attempts have been made to measure cytokine production in the supernatants of the cell cultures used in the LTT. Similarly, cytokine gene expression has been measured in the mRNA isolated from the PBMCs. The measurement of cytokines focuses more on the functionality of the immune response and might result in higher specificity. Similar to the proliferation assays, most studies have been performed in PBMCs isolated from nickel allergic subjects. The results are summarized in Appendix 2.

It has been shown that the assessment of IL-2 gene expression could be a specific marker for nickel allergy with a relative good sensitivity (Falsafi-Amin et al., 2000, 2008), whereas TNF- α and IL-4 expression were not useful as markers for nickel allergy (Falsafi-Amin et al., 2000). IL-2 plays an important role in the proliferation of T lymphocytes.

IFN- γ plays an important role in contact dermatitis, since it is involved in Th1-mediated immune responses. Rustemeyer et al. (2004) have measured IFN- γ and IL-5 in PBMCs cultured with or without cytokine cocktails. IL-5 was a more sensitive marker than IFN- γ to detect nickel allergy. When IL-5 was compared with the proliferation assay (LTT + Th2 cytokine cocktail) the sensitivity was similar. Hence, the measurement of IL-5 could be used to identify patients with nickel allergy, but the assay is

similar to the proliferation assay. Two other studies have shown that IFN- γ is not a good biomarker for nickel allergy (Borg et al., 2000 en Spiewak et al., 2007). In contrast, a recent study has shown that IFN- γ was a sensitive and specific marker for nickel allergy, whereas IL-10 could not be used as a marker. This study also demonstrated that both IL-10 and IFN- γ could not be used as biomarkers for palladium allergy (Bordignon, et al. 2009). In a study from Cederbrant et al. (2003), IL-10 was an accurate marker for nickel allergy, in contrast to the study of Bordignon et al. (2009). Finally, Minang et al. (2005) have shown that IL-4, IL-5, IL-10, IL-13, IFN- γ could not be used as individual biomarkers for nickel allergy. When the panel of cytokines was combined, the sensitivity improved. Furthermore, a strong correlation was demonstrated between cytokine responses and the severity of the patch test.

Besides nickel, not many other other contact sensitizers were used to assess if cytokines could be used as markers for contact dermatitis. For potassium dichromate, it has been shown that IFN- γ was a moderate biomarker, with a sensitivity of 74% and a specificity of 72% (Trattner et al., 2003). Moed et al. (2005) have investigated if IFN- γ and IL-5 could be used as biomarkers for different types of contact dermatitis. They have shown that it was dependent on the contact allergen which cytokine could be used. IFN- γ was a good biomarker for nickel, fragrances, and PPD, whereas IL-5 was a good biomarker for nickel and chromium. Both cytokines could not be used to diagnose cobalt allergy. In another study, IL-4, IL-2, IL-13, and IFN- γ were measured and for the diagnosis of nickel allergy IL-4 and IL-13 were reliable biomarkers. For all other metals (cobalt, chromium, palladium, gold), none of the cytokines could be used to assess contact allergy. It was only possible to detect palladium allergy with a moderate sensitivity when all four cytokines were used (Minang et al., 2006).

3.4 LTT: gene expression profiles

There is one study that measured gene expression profiles in PMBCs that were cultured with chromium in order to find potential biomarkers for chromium allergy. Gene expression profiles were determined by microarray technology and this was done in a small group of patients with chromium allergy (n=3) and 3 controls. A total of 26 genes were differently expressed between patients and controls, 18 were upregulated and 8 were downregulated. These genes were involved in immunological or inflammatory processes, cell growth, apoptosis, metabolism and cell communication. The diagnostic value of three selected genes was tested in 7 patients and 3 controls by using RT-PCR. The most sensitive gene was *CASP8*, which is involved in apoptosis. The other two genes: *EST2* (cell growth) and *CISH* (cell communication) could only identify patients with a high proliferation in the LTT assay. It is unknown what the sensitivity and the specificity of these genes is compared to the patch test (Hansen et al., 2005).

4 Discussion

In this letter report an inventory of *ex vivo* methods for the assessment of sensitization to LMW chemicals in exposed people is described. Such an *ex vivo* method is less invasive than the patch test or the skin prick test that can be used to detect type IV or type I mediated immune responses, respectively. Furthermore, there is no risk on active sensitization when an *ex vivo* method is used.

The LTT has been the method of choice in most studies and cell proliferation is most commonly used as a read-out, followed by cytokine production or cytokine gene expression. The majority of studies have used the LTT for the detection of nickel allergy, whereas only a limited number of studies included patients with contact dermatitis induced by other metals or chemicals. The sensitivity and specificity of the LTT assays for nickel varies between the studies. One aspect that makes it difficult to compare these LTT assays is that there is no validated and standardized protocol. Hence, the different studies that conducted the LTT assay for nickel have used different protocols, including culture differences such as the nickel concentrations, the culture time and the number of cultured PBMCs. Furthermore, the cut-off value that is used to distinguish between a positive and a negative test differs, some studies use a stimulation index (SI) of 2, whereas others use a SI of 3. In conclusion, the lack of a standardized protocol makes it impossible to compare the LTT assays for nickel. Besides that, the outcome is influenced by the variability that exists between different patients. Not all patients have PBMCs that respond to the contact allergen. Probably, in these patients the memory T lymphocytes reside in the skin and only a limited number circulates in the blood. Furthermore, the *in vitro* system uses PBMCs, which are T lymphocytes (>80%) and monocytes (<20%). In this assay, the monocytes serve as antigen-presenting cells. However, in the *in vivo* situation the antigen is presented by Langerhans cells which are more efficient in antigen presentation than monocytes (Martins et al., 2008).

There are only a limited number of studies that have used the LTT for other metals. The LTT could not be used for the detection of cobalt, gold and palladium allergy, since either the sensitivity or the specificity of the assays was low. The MELISA, which is a slightly modified LTT assay, has also been used for gold and palladium but the sensitivity for gold was low, whereas the specificity for palladium was only moderate. Chromium has been tested in a number of studies and the LTT has a moderate sensitivity in most studies, except in the study of Moed et al. (2005), in which the LTT failed to identify patients with chromium allergy. In this study, the sensitivity for chromium improved when the LTT was modified by adding cytokine cocktails, but was still low. Other contact sensitizers were tested as well in this study and it was shown that the LTT could not identify patients with contact dermatitis for cobalt, PPD, fragrance mix and MCI. The addition of cytokine cocktails improved the sensitivity of the LTT for PPD from low to moderate (Moed et al., 2005). In general, the addition of cytokine cocktails improves the LTT in almost all studies, but the best results are obtained for nickel and PPD and for other contact sensitizers the sensitivity of the LTT is still low (Rustemeyer et al., 2004, Moed et al., 2005, Spiewak et al., 2007). In two small studies it was shown that the LTT was a sensitive and specific assay to detect allergy for DNCB (Levis et al., 1976) and thiurams (Kimber et al, 1991). These results were obtained in a limited number of patients and it is unknown if these data can be extrapolated to a larger group of patients. Many studies that have used the LTT for this purpose use small groups of patients and it is unknown if these group sizes provide sufficient statistical power.

From this survey it can be concluded that the LTT has been predominantly used for nickel. The limited number of studies with other contact sensitizers might be explained by differences in the mechanisms of T cell activation between nickel and other contact sensitizers. Nickel can directly activate T cells, whereas most other contact sensitizers have to bind to proteins first to become immune reactive.

Probably it is possible that a chemical can bind to a protein in the *in vitro* system. Also, in some studies the chemical has been coupled to a protein before adding it to the cell culture. Furthermore, many chemicals need to be oxidized before they are able to bind to proteins and metabolic activity is limited in the *in vitro* system. This can be overcome by adding either metabolic enzymes to the cell culture or by using the reactive metabolite instead of the chemical itself. Hence, for other contact sensitizers the protocol for the LTT is more complicated than for nickel. There were no studies that have used the LTT for respiratory sensitizers.

A drawback of the proliferation studies has been that the test sometimes fails to distinguish between patients and controls. This might be a methodological problem, since some studies show more promising results. To overcome this, more recent studies have focused on the functionality of the immune system and have measured cytokines as possible biomarkers. Again, in almost all studies this has been done for nickel allergy. It seems that IL-2 might be a good biomarker for nickel allergy (Falsafi-Amin et al, 2000, 2008), but this is tested in a limited number of patients. For IL-4, IL-5, IL-10, IL-13 and IFN- γ the results of the different studies are inconsistent (Falsafi-Amin et al, 2000, Cederbrant et al, 2003, Rustemeyer et al, 2004, Minang et al, 2005, Bourdignon et al, 2008, Borg et al. 2000, Spiewak et al, 2007). The addition of Th2 cytokine cocktail to the LTT might improve the sensitivity of the assay as has been shown by Rustemeyer et al (2004). A few studies demonstrate that IL-5 might be a good biomarker (Rustemeyer et al, 2004, Spiewak et al, 2007). The data for other contact sensitizers is limited. Palladium allergy could not be detected with IL-10 and IFN- γ (Bordignon et al, 2008). IFN- γ could be a biomarker for chromium allergy (Trattner et al, 2003). Minang et al. (2006) tested different contact sensitizers and have shown that the best results were obtained for nickel and especially IL-4 and IL-13 were good markers. Cobalt and gold allergy could not be detected with cytokine measurements. For palladium and chromium the best sensitivity was obtained when all cytokines were measured. Moed et al. (2005) have measured IFN- γ and IL-5 and demonstrate that IL-5 was a good marker for nickel and chromium allergy, whereas IFN- γ was a good marker for nickel, fragrances, PPD and MCI. A drawback of this study was that it was performed in a limited number of patients.

The possibility to use gene expression profiles to find relevant genes that can be used to detect chromium allergy was explored in one study (Hansen et al., 2005). Such an approach offers the opportunity to identify diagnostic biomarkers. Although it was possible to discriminate between patients and controls this was done only in a limited number of subjects which makes it difficult to interpret if the changes in gene expression are relevant for contact dermatitis in general or for chromium allergy. Such an approach could result in relevant biomarkers, but it is important to test this in a large number of patients and controls and to include different types of contact allergens to find out if the identified genes are specific for one contact allergen or that they are related to more general processes that are involved in immune responses.

The advantages of an *ex vivo* method for the detection of sensitization by LMW chemicals, such as the less invasive nature of the test and the absence of the risk for active sensitization, are clear. However, at the moment these alternative methods are in an experimental phase and are predominantly tested for nickel, whereas it is unknown if allergies for other contact or respiratory sensitizers can be assessed *ex vivo* as well. It is important that the accuracy of the assay is the same or better than the patch test. However, although many studies report on the occurrence of false-positive and false-negative findings in the patch test, the exact sensitivity and specificity is unknown. Rustemeyer et al. (2004) have shown that the patch test for nickel has a sensitivity of 54%, which is lower than the sensitivity of the LTT for nickel that was found in most studies. The specificity of the patch was high in this study: 96%. In order to judge the reliability of an *ex vivo* method, insight in the accuracy of the patch test is necessary.

One important aspect that needs to be emphasized is that until now, none of the LTT studies have been validated extensively. An *ex vivo* method for the assessment of sensitization to contact or respiratory sensitizers will only be successful when an optimized and standardized protocol has been developed, that has been validated in an intralaboratory validation study with sufficient patients and controls. The addition of cytokine cocktails could possibly improve such a method, and this should be taken into account during validation. Furthermore, for contact and respiratory sensitizers other than metals, the most optimal conditions should be experimentally elucidated, i.e. requirements for protein binding and metabolism. Finally, an integrated approach in which several parameters are assessed, such as proliferation, cytokine production or gene expression might lead to an optimal *ex vivo* assay. Such a method could be used in a preclinical stage to identify workers that are sensitized to a chemical. In sensitized persons, additional diagnostic testing could be performed to confirm sensitization and measures should be taken to prevent the development of allergy.

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Appendix 1: LTT: cell proliferation or MELISA

Compound	Population	N=	Sensitivity ^a	Specificity ^b	Comments	Reference
LTT						Macleod 1970
Nickel	Patients	12	58%	100%		
	Controls	14				
Nickel	Patients	8	87.5%	81%	LTT was less sensitive than the patch test	Svejgaard., 1978
	Controls	16				
Nickel	Patients	14	76%	90%		Silvennoinen-Kassinen, 1981
	Controls	10				
Nickel	Patients	21	86%	91%	LTT was less sensitive than the patch test	Rasanen & Tuomi, 1992
	Controls	23				
Nickel	Patients	0	--	11%		Lisby, 1999
	Controls	18				
Nickel	Patients	5	100%	0%		Cederbrant, 2003
	Controls	5				
Nickel	Patients	7	71%	86%	Specificity increased when proliferation was combined with measurement of IL-2 production (Appendix 2)	Falsafi-Amin 2000
	Controls	7				
Nickel	Patients	14	--	--	LTT + cytokine cocktails (IL-7+IL-4 of IL-7+IL-12). It was not possible to calculate sensitivity and specificity. Correlation with the patch test was assessed. The correlation between LTT and patch test increased when IL-7 + IL-4 were added: from 0.61 (LTT) to 0.74 (LTT + IL-7 +IL-4). IL-7 + IL-12 did not affect the correlation.	Spiewak, 2007
	Controls	14				
Nickel	Patients	8	100%	80%		Kimber, 1990
	Controls	10				
Nickel	Patients	31	63%	75%	LTT	Rustemeyer, 2004
			77%	64%	LTT + cytokine cocktail (IL7 + IL-12)	
			91%	72%	LTT + cytokine cocktail (IL7 + IL-4)	
	Controls	38	The sensitivity of all LTT assays is higher than sensitivity patch test, which was 54%.			

Compound	Population	N=	Sensitivity^a	Specificity^b	Comments	Reference
Nickel	Patients	7	86%	100%	LTT	Moed, 2005
			86%	86%	LTT + IL-7/IL-12	
			71%	86%	LTT + IL-7/IL-4	
Chromium	Patients	7	0%	100%	LTT	
			43%	100%	LTT + IL-7/IL-12	
			43%	100%	LTT + IL-7/IL-4	
Cobalt	Patients	7	--	--	LTT	
			14%	100%	LTT + IL-7/IL-12	
			0%	100%	LTT + IL-7/IL-4	
Fragrance mix	Patients	7	0%	--	LTT	
			14%	100%	LTT + IL-7/IL-12	
			29%	100%	LTT + IL-7/IL-4	
PPD ^c	Patients	7	17%	100%	LTT	
			71%	100%	LTT + IL-7/IL-12	
			43%	100%	LTT + IL-7/IL-4	
MCI	Patients	7	-	--	LTT	
			29%	100%	LTT + IL-7/IL-12	
			43%	100%	LTT + IL-7/IL-4	
	Controls	7				
Cobalt	Patients	14	43%	89%		Veien & Svejgaard, 1978
	Controls	9				
Chromium	Patients	8	75%	100%		Rasanen, 1991
	Controls	8				
Chromium	Patients	20	65%	95%		Martins, 2008
	Controls	20				
Chromium	Patients	24	62.5%	91%		Christiansen, 1980
	Controls	11				

Compound	Population	N=	Sensitivity^a	Specificity^b	Comments	Reference
Chromium	Patients	31	65%	92%		Al-Tawil, 1983
	Controls	24				
Chromium	Patients	37	70%	70-74%		Lindemann, 2008
	Sensitized but no complaints	19				
Isothiazolinone	Patients	18	50%	100%		Stejskal, 1990
	Controls	16				
Thiuram	Patients	4	100%	100%		Kimber, 1991
	Controls	3				
PPD	Patients	13	85%	--	No control group	Skazik, 2008
	Controls	0				
PPD ^c	Patients	11	100%	88%	It is necessary to use Bandrowski's base to achieve a sensitivity of 100%	Sieben, 2002
	Controls	8				
DNCB	Patients	4	100%	100%		Levis, 1976
	Controls	7				
Fragrances	Patients	32	31.3%	--	No control group	Sieben, 2001
Oak moss	Patients	5	100%	--		
Isoeugenol	Patients	5	80%	--		
Geraniol	Patients	2	50%	--		
Hydroxycitronellal	Patients	3	67%	--		
Cinnamic aldehyde	Patients	1	0%	--		
α -Amyl-cinnamic aldehyde	Patients	1	0%	--		

Compound	Population	N=	Sensitivity^a	Specificity^b	Comments	Reference
Nickel	Patients	19	82%	17%		Cederbrant, 1997
	Controls	12				
Gold	Patients	20	70%	58%		
	Controls	14				
Palladium	Patients	18	82%	53%		
	Controls	16				
MELISA						Cederbrant, 1997
Nickel	Patients	19	95%	25%	Compared to the LTT: the MELISA is less specific for nickel and less sensitive and specific for gold. The MELISA is more sensitive and specific for palladium.	
	Controls	12				
Gold	Patients	20	55%	79%		
	Controls	14				
Palladium	Patients	18	94%	69%		
	Controls	16				
Nickel	Patients	15	100%	60%		Valentine-Thon & Schiwara, 2003
	Controls	10				

Abbreviations: MCI: methyl-chloro-isothiazolinone; PPD: *p*-phenylene-diamine; DNCB: dinitrochlorobenzene; DNBSO₃: dinitrobenzene sulphonic acid; ^aSensitivity = number of patients correctly identified / total number of patients x 100%; ^b Specificity = number of controls correctly identified in the test / total number of controls x 100%; ^cLTT was performed with either PPD or Bandrowski's base, de reactive metabolite of PPD

Appendix 2: LTT: cytokine production or expression

Compound	Population	N=	Cytokines	Sensitivity ^a	Specificity ^b		Reference
Cytokines							
Nickel	Patients	5	IL-10	100%	80%	No correlation with the severity of the patch test	Cederbrant, 2003
	Controls	5					
Nickel	Patients	7	IL-2	71%	100%		Falsafi-Amin, 2000
			TNF- β	57%	71%		
			IL-4	43%	86%		
Controls	7						
Nickel	Patients	7	IL-2	86%	100%	.	Falsafi-Amin, 2008
	Controls	7					
Nickel	Patients	31	IFN- γ	63%	75%	LTT + IL7 + IL-12	Rustemeyer, 2004
			IL-5	61%	4%	LTT	
			IL-5	92%	75%	LTT + IL7 + IL-4	
			In vitro test is more sensitive than the patch test (54%) but less specific (96%)				
			Controls	38			
Nickel	Patients	30	IL-4	63%	100%	Correlation with the severity of the patch test	Minang, 2005
			IL-5	33%	100%		
			IL-13	57%	100%		
			IL-10	37%	100%		
			IL-13	63%	100%		
			IFN- γ	37%	100%		
			All	77%	100%		
			Controls	10			
Nickel	Patients	20	IL-10	0%	0%		Bordignon, 2008
			IFN- γ	100%	100%		
Palladium	Patients	20	IL-10	95%	0%		
	Controls	10	IFN- γ	50%	100%		

Compound	Population	N=	Cytokines	Sensitivity ^a	Specificity ^b	Reference
Nickel	Patients	35	IL-4	--	--	No data on sensitivity and specificity. IL-4 and IL-5 can distinguish between patients and controls. IFN- and TNF- are not useful.
	Controls	30	IL-5 IFN- γ TNF- α			
Nickel	Patients	14	IL-2	--	--	No data on sensitivity and specificity but correlation with the patch test. Without cytokine cocktails the correlation is below 0.5, The best results were IL-13 measured in the LTT with IL-7+IL-4 (0.65), followed by IL-5 measured in the LTT with IL-7+IL-4 (0.55) and IL-2 measured in the LTT with IL-7+IL-12 (0.54). IFN- was not a reliable marker.
	Controls	14	IL-13 IL-5 IFN- γ			
Potassium dichromate	Patients	20	IFN- γ	74%	72%	Trattner, 2003
	Controls	30				
Nickel	Patients	7	IFN- γ	86%	100%	Moed, 2005
			IL-5	100%	86%	
Chromium	Patients	7	IFN- γ	57%	86%	
			IL-5	100%	86%	
Cobalt	Patients	7	IFN- γ	57%	100%	
			IL-5	57%	71%	
Fragrance mix	Patients	7	IFN- γ	86%	100%	
			IL-5	57%	71%	
PPDc	Patients	7	IFN- γ	100%	100%	
			IL-5	57%	86%	
MCI	Patients	7	IFN- γ	86%	71%	
			IL-5	57%	86%	
	Controls	7				

Compound	Population	N=	Cytokines	Sensitivity ^a	Specificity ^b	Reference
Nickel	Patients	17	IL-4	82%	100%	Minang, 2006
			IL-13	94%	100%	
			IL-2	76%	100%	
			IFN- γ	71%	100%	
			All	100%	100%	
Cobalt	Patients	12	IL-4	38%	100%	
			IL-13	23%	100%	
			IL-2	33%	100%	
			IFN- γ	15%	100%	
			All	46%	100%	
Chromium	Patients	9	IL-4	22%	100%	
			IL-13	56%	100%	
			IL-2	22%	100%	
			IFN- γ	0%	100%	
			All	67%	100%	
Palladium	Patients	4	IL-4	50%	100%	
			IL-13	25%	100%	
			IL-2	25%	100%	
			IFN- γ	0%	100%	
			All	75%	100%	
Gold	Patients	10	IL-4	10%	100%	
			IL-13	20%	100%	
			IL-2	50%	100%	
			IFN- γ	10%	100%	
			All	50%	100%	
	Controls	5				

* Cytokine production or expression was detected by ELISA, ELISPOT or PCR

^aSensitivity = number of patients correctly identified / total number of patients x 100%; ^b Specificity = number of controls correctly identified in the test / total number of controls x 100%;