Interactions of Chemicals and Metal Ions with Proteins and Role for Immune Responses

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Abstract: Chemicals and metal ions often induce allergic contact dermatitis. We review here recent advances in the development of *in vitro* assays for prediction of skin sensitizing potency based on chemical and biological reactivity as well as in the identification of physiological binding partners and immunological pathomechanisms of chemical and metal ion induced disease.

Keywords: Allergic contact dermatitis, skin sensitization, chemical, metal ion, hapten, arnica, nickel, proteomics.

INTRODUCTION

Chemicals of low molecular weight (less than 500 Da) and metal ions interact with proteins by covalent or non-covalent binding. As a consequence, innate immune responses are directly activated by poorly understood mechanisms and antigenic determinants are generated which trigger adaptive T and/or B cell dependent immunity. The result is often the development of T cell mediated allergic contact dermatitis (ACD), which is one of the most frequent occupational dermatoses [1-3]. About 15-20% of the population is sensitized to a least one substance. 5 to 10% develop ACD at least once a year, and over 3000 substances are known to cause ACD [2,3]. In order to understand why some chemicals and metal ions are acting as potent skin sensitizers while others are harmless we have to learn more about the efficiency of skin penetration, uptake and metabolization, as well as the mechanisms of protein binding based on chemical structure, reaction mechanisms as well as the biological consequences. Various assays are currently used to assess skin sensitizing potency of pure substances. The ultimate goal of research in this area is the development of reliable *in vitro* test systems that will allow the identification and classification of potential skin sensitizers based on characteristic physicochemical and biological features. The definition of predictive parameters for risk assessment will lead to improved product safety by appropriate labeling, the exclusion of potentially hazardous compounds from consumer products, and the avoidance of experimental animal use. Combined efforts in this area and in basic immunological research will help to close the big gap of knowledge about the physiological target structures of haptens and metal ions. Therapeutic interference with the initial chemical-protein interactions could help to prevent or treat ACD more specifically.

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CHEMICAL INTERACTION WITH PROTEINS

We are exposed daily to a large number of chemicals, e.g. in food, drugs, cosmetics, plants or in our work environment. When entering our body via mucosal surfaces or skin, reactive chemicals, so-called haptens, can covalently modify proteins by various reaction mechanisms, e.g. nucleophilic substitution, Michael-type addition or radical reactions. Non-reactive pro-haptens can be metabolized in tissues like skin to yield reactive haptens. However, little is known about their metabolic processing in the skin [4] or the physiological binding partners for chemicals and metal ions and the molecular consequences of chemical modification of target proteins.

Several outcomes are possible (Fig. **1**): alteration of protein function due to conformational changes or interference with functional domains, alteration of protein localization and alteration of protein-protein interactions, generation of antigenic determinants for T or B lymphocytes etc. The end result of these interactions can be allergies, adverse drug reactions or autoimmune disease.

TEST SYSTEMS TO EVALUATE THE SKIN SENSITIZING POTENCY OF CHEMICALS

Several assay systems have been developed in order to assess the skin sensitizing potency of chemicals, i.e. the potential to sensitize people for the development of allergies.

(1) *In Vitro* Assays

Quantitative Structure Activity Relationship (QSAR) studies give information about the correlation of chemical structure, potential sites of reactivity with skin sensitizing potency [5,6]. Recently, QSARs are being refined by including simulation of skin metabolism and a classification based on the reaction types of the compounds of interest [7,8]. Chemical-protein interaction is often determined by measuring the reactivity of a chemical with model proteins or peptides such as glutathione [9].

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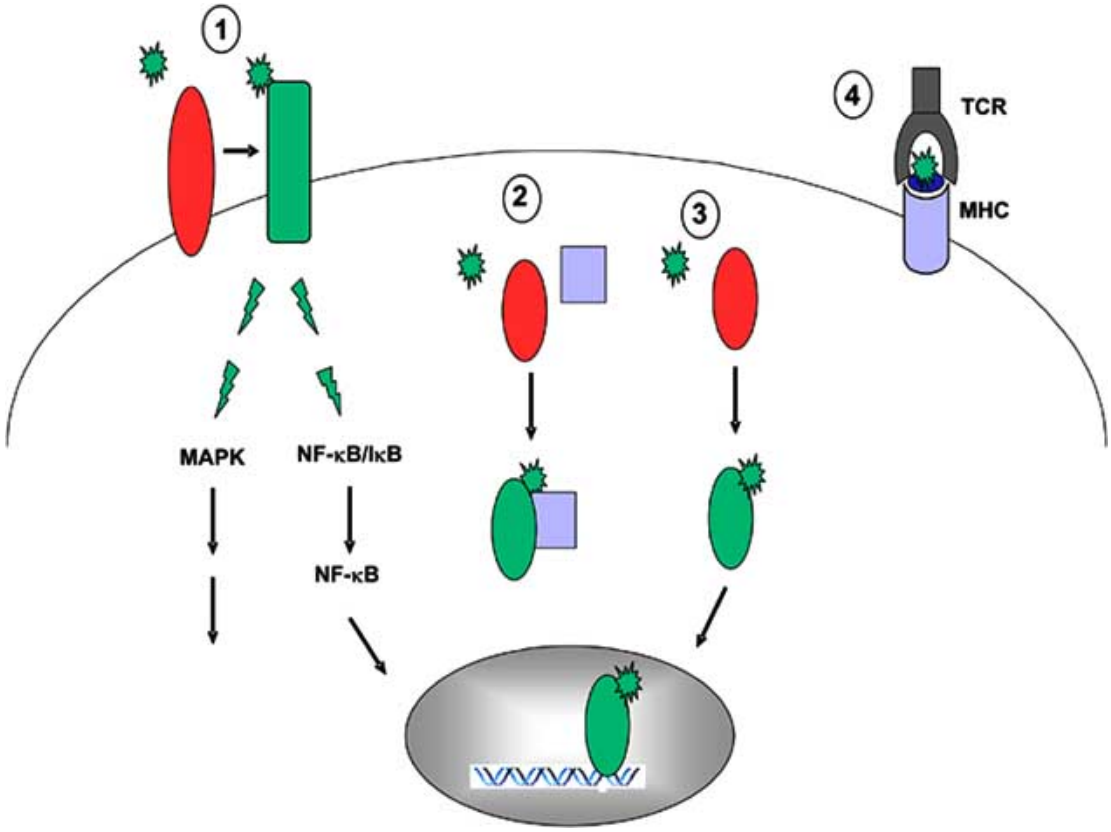


Fig. (1). Hypothetical and known consequences of chemical interaction with proteins.

Chemical modification of proteins, e.g. receptor kinases, may result in a conformational change leading to activation or inhibition of signaling function (1), changes in protein-protein interactions (2) or alteration of cellular localization of proteins, e.g. the translocation of transcription factors into the nucleus and promoter activation (3). Moreover, the generation of chemically modified MHC-associated peptides or the chemical- or metal ion-induced TCR-MHC peptide interaction results in the activation of chemical-reactive T cells (4).

Cell-based assays focus on the use of the prototypic antigen presenting cells (APC) of the immune system, the dendritic cells (DC). Being sensors for danger signals such as infection, trauma or allergen challenge, DC become activated by these signals and migrate to draining lymph nodes. Following skin contact with allergens epidermal Langerhans cells and also dermal DC become activated in part by crosstalk with other cells like keratinocytes. After their migration, the activated DC present chemical-modified peptides or metal ions on MHC molecules to naïve T cells in the lymph node and upon activation also induce skin-specific homing receptors, which direct the effector T cells specifically to the skin where they cause ACD [1,10-12]. Hallmarks of DC interaction with contact sensitizers are upregulation of MHC class II molecules, costimulatory molecules such as CD40, CD80 or CD86 and of cytokines such as IL-1β, TNF-α or IL-12 or chemokines like CXCL10 [13-19] by largely unknown mechanisms. These allergen induced changes are measured by flow cytometry of surface molecules or intracellular cytokines, as well as ELISA and ELISPOT techniques for detection of cytokines and chemokines. Recommendations for the appropriate use of DC for allergen testing have recently been published by experts in the field [20].

Since allergens themselves seem to polarize cytokine patterns of DC and T cells in a pro-allergenic fashion [21-23], cytokine profiling of DC and T cells may turn out to be a useful tool for the characterization of allergens. More recently, gene expression profiling of DC is being tested as a tool for the identification of skin sensitizers [24] and proteomic approaches are underway to identify physiological binding partners of chemicals and metal ions [25-27]. Furthermore, the use of engineered skin equivalents is promising [28].

(2) *In Vivo* Assays Using Animals

In vivo assays using animals will be more and more restricted by legislation and will therefore have to be replaced by appropriate *in vitro* assays for skin sensitizing potency and risk assessment. Currently, the Local Lymph Node Assay (LLNA) adopted by the OECD as guideline 429 as only stand-alone test method for skin sensitization [5,29,30] is used to determine skin sensitizing potency. In this assay, chemicals are classified as weak, moderate or strong sensitizers based on their potency to induce cell proliferation in draining lymph nodes following sensitization via ear skin of mice. Other assays such as the guinea pig maximization test have great limitations due to the experimental setup and are therefore of limited value for potency evaluation [30].

It has become clear that skin sensitizing potency does not necessarily correlate with the potency to eventually induce ACD. Many parameters which vary individually influence elicitation thresholds, e.g. skin penetration and chemical reactivity which influence bioavailability or the duration and site of exposure [30]. The important role of bioavailability in dependence of the solvent used in the LLNA was impressively demonstrated recently. Thus, the classification of a given contact sensitizer can change significantly when the solvent used in the LLNA is changed [19]. Furthermore, the LLNA has also not been designed to test preparations, but only pure substances.

All of these assays aim at the reliable prediction of skin sensitizing potency eventually without the use of animals and provide a basis for appropriate labeling of consumer products and avoidance of potential sensitizers. However, for improvements in the therapy of ACD, it will be extremely helpful to identify the complex array of factors that eventually lead to the development of ACD in some, but not other sensitized individuals.

CHEMICAL INTERACTION WITH SKIN ACTIVATES THE INNATE IMMUNE SYSTEM

It is a prerequisite for skin sensitization to chemicals that they activate keratinocytes, epidermal Langerhans cells (LC) [1] and, as recently shown, dermal DC [31,32]. One of the striking features of contact sensitizers is their capability to activate the innate immune system in a way similar to pathogen associated molecular patterns (PAMPS) of bacteria and viruses, ligands for Toll-like receptors (TLR) [33,34]. Like PAMPS, the induction of inflammation, the so-called irritant effect of contact sensitizers, leads to DC activation by triggering of signaling cascades, upregulation of costimulatory molecules and induction of cytokine and chemokine production by DC and keratinocytes in the skin. The activated DC then migrate to the draining lymph nodes where they activate the adaptive immune system, i.e. T cells [1]. These initial events are crucial for efficient sensitization, but also provide sufficient cytokines and chemokines upon elicitation to allow for skin homing of circulating effector T cells from the blood [1]. It has recently been shown that contact sensitizers analogous to type I PAMPS [35] can polarize cytokine patterns to a type I response, which is required for ACD [21-23]. However, it is unclear whether this action of contact sensitizers involves TLR. One of the events induced by organic chemicals is the activation and translocation of p38 MAP kinase (MAPK), the activation of the ERK1/ERK2 MAPK and of the transcription factor NF-κB [36-39]. Interestingly, DC activation seems to require hapten interaction with thiol groups resulting in activation of ERK1/2 MAPK while reaction with amino groups activates p38 MAPK [39,42]. In fact, thiol antioxidants present during hapten stimulation of DC were able to block tyrosine phosphorylation and activation of MAP kinases by several contact sensitizers and protein binding by TNCB [25]. An interesting observation was that DNCB and $NiSO_4$ differ in their capacity to activate members of the three different MAPK families or NF-κB, suggesting that different pathways and different target structures for the activation of DC and other cell types exist for organic chemicals and metal ions [39-41,43]. It is at present unknown how chemicals activate these signaling pathways but the covalent binding to thiol and amino groups seems to play a crucial role. It remains to be determined whether chemical protein modifications directly alter the signaling function of potential target proteins like kinases or act via other mechanisms (Fig. **1**). The elucidation of these mechanisms should provide us with novel tools for therapeutic interference in ACD.

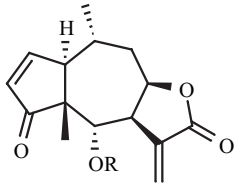
CHEMICAL MODIFICATION OF PROTEINS GENERATES ANTIGENIC T CELL DETERMINANTS

Following the activation of the innate immune system, antigen is presented to naïve T cells in the local draining lymph nodes by DC immigrated from skin following their activation by contact sensitizers. For a number of chemical allergens it has been clearly shown that they generate hapten-modified peptides that are presented and recognized by $CD8^+$ or $CD4^+$ T cells on MHC class I and class II molecules, respectively. Two examples of hapten-modified peptide generation are the binding of reactive 2,4-dinitro- (DNP) or 2,4,6-trinitrophenyl (TNP) compounds to ε-amino groups of lysine residues and the binding of reactive β-lactam antibiotics like penicillins to such amino groups [1]. Binding to thiol groups is also relevant for such haptens as clearly shown for sesquiterpene lactones [44]. For nickel, different modes of coordinative binding to MHC and/or MHC bound peptides and the TCR have been shown to be relevant in the activation of Ni-specific T cells [45,46]. Other chemicals seem to directly connect MHC and TCR without covalent binding [47]. The end result is the activation of chemical-reactive T cells which can cause allergy, autoimmunity or adverse drug reactions.

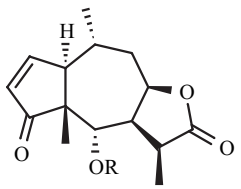
SESQUITERPENE LACTONES AS IMMUNO-SUPPRESSANTS AND ALLERGENS

Sesquiterpene lactones (SLs) belong to those secondary plant metabolites that are often reported to cause ACD [48]. These C-15 terpenoids with a characteristic unsaturated lactone moiety are the active constituents of many medicinal plants from the Asteraceae family. A prominent example is the European *Arnica montana* alcoholic preparations of which have been approved by the German Commission E and the European Scientific Cooperative on Phytotherapy (ESCOP) for topical treatment of various inflammatory diseases [49]. SLs from the pseudoguaianolide type, such as helenalin and 11α,13-dihydrohelenalin and their ester derivatives (Fig. **2**) which constitute about 0.6% of the dry mass of Arnica flowerheads and 0.06% of the tinctures extracted from them [50], are the main ingredients with anti-inflammatory activity.

It has been shown in various *in vitro*, *ex vivo* and *in vivo* assays and in clinical trials that Arnica preparations as well as the purified SLs possess antiinflammatory properties [49]. We have recently demonstrated that alcoholic Arnica preparations as well as their isolated SLs influence inflammation at a very central point by inhibiting DNA binding of the transcription factor NF-κB, a central mediator of the human immune response [51-54]. Genes that are regulated by NF-κB include proinflammatory and



Helenalin (R = H) and ester derivatives
R = H or short chain fatty acids



11α,13-Dihydrohelenalin (R = H) and ester derivatives
R = H or short chain fatty acids

Fig. (2). Sesquiterpene lactones in flowerheads from *Arnica montana.*

inflammatory cytokines such as interleukin-1, -2, -4, -6 and -8 or TNF-α, as well as genes encoding immunoreceptors, cell adhesion molecules, acute phase proteins, and enzymes such as cyclooxygenase. Moreover, NF-κB is involved in the regulation of genes required for antigen presentation on the cell surface, e.g. MHC class I molecules, β_2-microglobulin, as well as the TAP-1 transporter required to transport peptides into the endoplasmic reticulum and the proteasome subunit LMP2 required for peptide generation [55]. In almost all cell types, NF-κB, composed of a p50 and a p65 subunit, is retained in an inactive cytoplasmic complex by binding to the inhibitory subunit IκB. Numerous pathways lead to the activation of NF-κB in which the TNF, TLR or the T cell receptor can be involved. All NF-κB activating signals converge on the IκB kinase, which phosphorylates IκB-α targeting it for ubiquitination and degradation. NF-κB translocates to the nucleus where it stimulates transcription [56-58].

We have provided evidence that helenalin and 11α,13-dihydrohelenalinacetate inhibit DNA binding of NF-κB in cell cultures most probably by alkylating its p65 subunit at Cys38. Although a slight inhibition of IκB degradation was also observed, we determined that this effect is secondary to the alkylation of p65 [51,53]. There are strong indications that this is a general mechanism for SLs, which possess α,β- or α,β,γ-unsaturated carbonyl structures such as α-methylene-γ-lactones or α,β-unsubstituted cyclopentenones. These structural elements preferably react with nucleophils, especially sulfhydryl groups by a Michael-type addition [59].

Helenalin derivatives were ten-fold more active in NF-κB inhibition than 11α,13-dihydrohelenalin derivatives. Accordingly, Arnica tincture which was prepared from flowers type Arbo representing the Central European type (here called Central European Arnica tincture) and dominating in helenalin derivatives was twice as active than tincture which was prepared from Spanish Arnica flowers (here called Spanish Arnica tincture) containing mostly 11α,13-dihydrohelenalin derivatives [54].

Using Arnica preparations as well as their isolated SLs we demonstrated that the production of NF-κB regulated cytokines and chemokines such as IL-1β, TNF-α and IL-8 was also reduced, even at lower concentrations than necessary for inhibition of NF-κB DNA binding. Again, isolated helenalin derivatives or Central European Arnica tincture exhibited a stronger NF-κB inhibitory activity compared to isolated dihydrohelenalin derivatives and Spanish Arnica tincture [54,60,61].

When investigating the inhibitory effect on IL-2 and on CD69 expression of SLs in lymphocytes, using whole blood a reduction in expression could be observed. However, the bifunctional SLs, possessing two α,β-unsaturated structural elements and with parthenolide as a model, showed a weaker activity than monofunctional substances with one α,β-unsaturated carbonyl group, such as 11α,13-dihydrohelenalin esters [62]. This different behaviour compared to other studies undertaken with cell cultures may be explained by their different bioavailability due to binding to human plasma proteins. We could demonstrate that helenalin esters are bound to a higher degree to human serum albumin (HSA) and blood plasma than the respective 11α,13-dihydrohelenalin derivatives and that SLs in the alcoholic preparations showed a lower degree of protein binding [63]. In accordance with these results penetration kinetics with pig skin as a model revealed a similar behaviour. Whereas isolated SLs permeated through the stratum corneum only in a very small amount, permeation of SLs was much higher when they were present in the tinctures. Again, 11α,13-dihydrohelenalinacetate showed a better penetration rate than helenalinisobutyrate [64].

All these studies demonstrate that SLs from Arnica possess an immunosuppressive effect by inhibiting production of various cytokines and chemokines, which seems to be contradictory to their property of triggering ACD. Thus, the already proven inhibition of IL-1β and TNF-α by SLs may reduce Langerhans cell migration from the epidermis to the dermis, an important event in the sensitization phase [1,65,66]. Moreover, SLs from Arnica may influence further important steps in the sensitization phase by reducing T cell activation shown by reduction of CD69, the earliest marker of activated $CD4^+$ and $CD8^+$ T cells and by reducing expression of IL-2, which is involved in proliferation, differentiation and activation of T cells [1,62,67]. Furthermore, SLs should also have a suppressive effect in the elicitation phase because of their cytokine and chemokine (e.g. IL-1β, TNF-α) suppressing effects. SLs directly inhibit DNA binding of NF-κB, it can be speculated that further events in the pathophysiology of ACD may be influenced. Up to now no studies on the molecular mechanisms of SLs in ACD have been carried out to reveal their paradoxical dual role as immunosuppressive as well as ACD causing agent.

The current concept in ACD is that SLs as those from *Arnica* function as haptens which combine in a Michael-type addition with a carrier, probably located at the skin level, to produce a complete antigen (Fig. **3**). The occurrence of an α-methylene-γ-lactone moiety is the most important structural element. Its hydrogenation, in the case of helenalin to the respective 11α,13-dihydrohelenalin derivative, results in a loss of allergenic activity, with the exception of 11α,13-dihydrohelenalinmethacrylate for which an allergenic

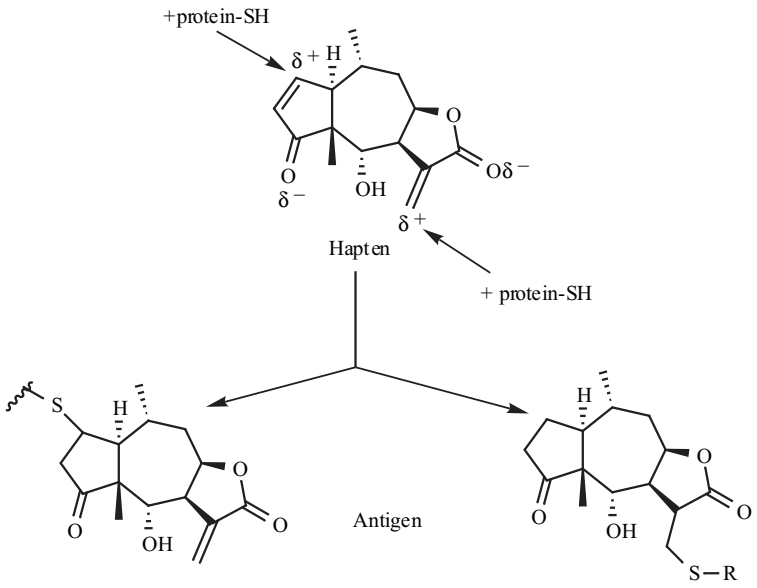


Fig. (3). Proposed reaction of helenalin (= hapten) with skin proteins in a Michael-type addition.

potential was also proven [68]. In the subsequent sensitization and elicitation phase Langerhans cells as well as $CD8^+$ and $CD4^+$ T lymphocytes are involved [1,69,70]. This proposed mechanism is discussed to be common for all SLs which possess an α-methylene-γ-lactone moiety [66,67].

ACD was studied in guinea pigs, which are especially suitable for weak sensitizers. The animals were sensitized with a 10% solution of acetone or a short ether extract prepared from the flowers of *A. montana*, followed by a challenge with helenalinacetate and helenalin. Evaluation based on the determination of the erythema indicated that both SLs studied showed a positive reaction, which was stronger for helenalin [71]. Moreover, the contact allergenic potency of some Arnica tinctures which were not studied for their qualitative and quantitative SL pattern has been reported [72]. According to these results the sensitization capacity of *Arnica montana* flowers as well as of its SLs was described as strong [71,72]. Up to now, however, no reports using mice, which are used for strong sensitizers such as TNCB [1], or the approved local lymph node assay (LLNA) [29,30] can be found in the literature to study or confirm the postulated high allergenic potential of Arnica SLs. One also has to keep in mind the often poor correlation of sensitzing potency and elicitation thresholds which vary individually [73].

Despite the postulated strong sensitization potency and the wide-spread use of Arnica preparations reports on ACD are rarely to be found when used as recommended. In his survey of Arnica allergy Hausen lists about 100 reported cases between 1844 and 1977, of which the majority were ascribed to the use of – often inexpediently undiluted – tincture of Arnica [72]. Further investigations underline the low incidence of ACD caused by Arnica, such as a recent Austrian study in which 5 of 443 consecutive patients (1.13%) were tested positive to Arnica. In another 6-year study with 3851 individuals tested, 118 reacted to the Compositae mix and 51 were positive to Arnica (about 1.32%) [74]. Nonetheless, it has to be kept in mind that the use of Arnica preparations can cause ACD (e.g. [75].

It remains to be determined whether the postulated strong skin sensitizing potency in which Arnica tinctures and SLs can be confirmed in mouse models and whether it correlates with the capacity to elicit CHS. Results from clinical studies seem to question the classification of SLs as strong sensitizers with respect to their capacity to elicit ACD. Therefore, a careful evaluation of the conditions by which allergic individuals have been sensitized including analysis of the conditions of exposure, genetic predisposition and immune status has to be included. This information and the elucidation of the molecular mechanism of ACD will be an interesting task by which valuable information can be provided for a safe use of herbal remedies and cosmetics. Moreover, reliable *in vitro* test systems are necessary to estimate the real sensitizing potency of natural compounds, such as SLs.

PROTEOMIC ANALYSIS OF NICKEL-PROTEIN INTERACTIONS

Transition metal nickel (Ni) represents the most frequent contact allergen in humans, affecting at least 15 to 20% of the population with a rising incidence, especially in females who have skin piercing [76,77]. The cause of nickel allergy may include possible genetic risk factors as shown by some familial clustering, but data of a recent study suggest that environmental, and not genetic factors, are of major importance for development of Ni-induced ACD [78]. Environmental factors have been defined in terms of potency of the hapten or metal, exposure concentration (dose/unit area), exposure duration, and the effect of injured epidermis [79]. However, to elucidate the disease underlying mechanisms, they have to be linked to allergen-specific cellular and molecular processes [80-82].

Cellular analysis includes the isolation of Ni-reactive $CD4^+$ and $CD8^+$ human T cell lines and clones from peripheral blood as well as from skin lesions of Ni-allergic patients. Such cells have been analysed for their MHC restriction, activation requirements, and potential essential contact sites likely involved in functional Ni binding

[45,46,83-86]. Moreover, it has been demonstrated that in healthy donors Ni-specific IL-10 producing, $CD25^+$, regulatory T cells may control the activation of both naive and effector T cells [86,87]. Together with other cellular studies, such metal-specific approaches may lead to the general development of new and improved diagnostic T cell assays in human ACD [88-90].

Arguing that the low Ni concentrations *in vivo* in skin will probably always exist in the molecular form of metal-protein complexes, we started searching for molecular metal-protein interactions potentially involved in the Ni-specific sensitization or elicitation phases. Since HSA is a prominent constituent in human sweat and skin and is able to cross the basal membrane, it appeared as a highly probable candidate to shuttle Ni ions to antigen presenting Langerhans cells in human epidermis. Furthermore, by addition of *in vitro* produced albumin-nickel complexes (HSA-Ni) to cell cultures, we observed that activation of Ni-specific human T cell clones could be induced either by classical addition of nickel salt solutions or equimolar metalloprotein concentrations, potentially also reflecting the hapten-like nature of Ni ions [91]. The known HSA Ni-binding site consists of a square-planar chelate structure in equilibrium with an octahedral form created by N-terminal amino acids, asp-ala-his [92,93]. Thus, according to our current model, transfer of Ni ions from shuttling HSA-Ni does imply TCR and MHC metal-binding sites with a higher affinity for Ni ions when compared to carrier ligand-binding sites, resulting in ion transfer and transient Ni-TCR-MHC complex formation with subsequent Ni-specific human T cell activation [27].

This and the search for molecules potentially involved in so far unknown Ni-epitope processing, prompted us to study Ni-protein interactions in human B cells as one representative type of APC [26]. As compared to whole B cell lysates only 28 Ni-interacting proteins were detected after Ni-affinity isolation and 2-dimensional electrophoresis (2-DE). Similar to the recently described human Cu and Zn metalloproteomes, a large number of high abundant proteins have been identified by mass spectrometry [26,94]. However, generally it has to be stressed that such metalloproteomes will vary from cell type to cell type, i.e. hepatocyte to B cell, as well as from species to species and may be highly dependent on the experimental setup chosen. Factors influencing such metal ion protein interactions include the ion size, radius and charge effect, preferential coordination geometry, the pH, and the selection by a specific liganding atom, dependent on the affinity of the a-group metal (hard) ions for oxygen-(O) donors and the affinity of the b-group metal (soft) ions for sulfur-(S) or nitrogen-(N) donors, together leading to differential metal-binding affinities to certain proteins [95]. Furthermore, interactions may be influenced indirectly by cooperative effects, e.g. the binding of a third partner to a molecule, like nitric monoxide (NO) to HSA, which induces a molecular conformational change thereby varying the copper – and potentially nickel – binding affinity to HSA [96]. In our most recent study [26] we could confirm known HSA-Ni binding [97], which was accompanied by a high loss of Ni-bead bound HSA during stringent washing steps, indicating a specific, but relatively low affinity binding. Low affinity binding implies that physiologically weaker, but potentially important, Ni-protein interactions may not have been detected in this study [26]. Furthermore, results obtained did support experimental evidence of recently described Ni-Cullin-2 protein interaction [98]. Cullin-2 represents a human homolog to the yeast cdc53 subunit of a ubiquitin ligase, which is involved in the control of cell cycle regulatory proteins, and has been shown to form a complex with an inactive transcriptional complex. The meaning of this association as well as the metal-protein interaction still remains to be elucidated.

In the same study, we unexpectedly identified a large number of heat shock proteins and chaperones as potential Ni-interacting proteins, which either interacted directly with Ni-NTA beads or indirectly, such as via CCT-specific substrates tubulin or actin still attached to the chaperone [26,99]. These proteins have been reported to play a central role in cell survival of eukaryotes and prokaryotes in response to a variety of physical and chemical stressors, including heavy metals [100-102]. Nickel sulfate, for example, has been demonstrated to induce hsp70 protein synthesis at non-cytotoxic concentrations in both human keratinocytes and fibroblasts [103]. In addition, elevated anti-Hsp70 serum concentrations have been associated with human Ni-allergy [104]. Since chaperonin containing CCT/TCP-1 is known to bind substrates in an ATP and Mg^{2+} dependent manner [105], we suggest that the nickel-protein interaction might interfere with the metal-binding site, thereby potentially influencing cytoskeletal folding of tubulin and actin [106]. To test whether additional Mg^{2+} and/or ATP concentrations affected the *in vitro* CCT alpha subunit identification after Ni-NTA affinity binding, high concentrations of Mg^{2+} and/or ATP were added during the affinity binding and Ni-protein interactions proven by Western blotting. There was no experimental difference detected, with or without Mg^{2+} and/or ATP, indicating a possible direct Ni-CCT subunit interaction. This prompted us to ask whether the whole hetero-oligomeric CCT complex, existing out of two stacked octameric rings, was enriched or just a subunit of it. We demonstrated that the whole chaperone machinery could be detected after Ni-NTA binding, implying enrichment of the whole cytosolic complex. To prove the CCT complex stability in the presence of high Ni concentrations, we added 1 mM nickel sulfate to whole cell lysates and analyzed the CCT complex integrity as before. No CCT complex dissociation was observed either with high concentrations of nickel sulfate nor imidazole indicating that potential chaperone machinery disassembly is not the reason for a known Ni-induced microtubule damage, whereby a functional abrogation cannot be excluded [106]. Furthermore, an indirect CCT binding via Ni-tubulin or Ni-actin interaction may also have contributed to Ni-NTA dependent enrichment. However, this seemed to be very unlikely for two reasons: 1) TCP-1 alpha was enriched independently of added ATP and Mg^{2+} concentrations *in vitro*, indicating a substrate independent Ni-CCT interaction analogous to an ATP-dependent substrate release described with luciferase and CCT [107], and 2), because most of the tubulin was detected outside of the complex and did not coelute with the entire complex, as determined with the technically sophisticated Blue Native/SDS two-dimensional PAGE system for analyzing oligomeric high molecular weight complexes [108].

Remarkably, both potentially Ni-interacting proteins Hsp70 and CCT are functionally involved in immune regulation. These proteins not only operate in molecular protein folding, protection and transport, but also bridge constitutive and inducible danger signals [99,109]. Hsp70, for example, which is released by dying cells, is able to activate dendritic cells and macrophages, presumably - like some other Hsps - *via* TLR [110,111]. Moreover, chemical inducible hexadecameric complex CCT, also called TriC, seems to play a key role in the MHC I antigen processing pathway by binding proteolytic intermediates in the cytosol, thereby protecting them from degradation [112].

Mass spectrometric data also indicated Ni-protein interactions to two cytoskeletal proteins, tubulin and actin, which have long been considered as substrates of TriC/CCT [26]. For tubulin, the view of direct metal-protein interaction was supported by further experimental data, suggesting a monomeric binding. Actually, Ni-tubulin interaction has been observed earlier and seems to be accompanied by conformational tubulin alterations and associated with Ni-induced cell injury and toxicity, promoting dramatic effects in the organization of microtubules [106,113]. Moreover, direct cationic Ni-interference with actin polymerization has also been detected and associated with *in vitro* conformational changes in the actin molecule, due to Ni-binding to the single high-affinity site for divalent cations [114]. Rapid signaling-dependent cytoskeletal reorganization has also been found, when Ni was added to platelets [115]. Remarkably, expression of a specific embryonic actin isoform has been observed in Ni-induced rat rhabdomyosarcomas as well as in human rhabdomyosarcomas, demonstrating the potential significance of malignant Ni effects on the cytoskeleton [116,117].

Another Ni-interacting candidate protein Erp44 is a recently discovered novel stress-inducible member of the thioredoxin family residing in the ER, and containing sequence features of a molecule probably involved in the control of oxidative protein folding [26,118]. This view is supported by the fact that Erp44 interacts directly with luminal Ero1-L alpha glycoprotein, which continuously and selectively re-oxidizes protein disulfide isomerase (PDI), one of the key proteins in the control of disulfide bond formation [119,120]. Both mechanisms, oxidative protein folding as well as disulfide isomerisation, are obligatory for correct protein conformation and the biological activity of many secreted and membrane proteins, such as immunoglobulins (Ig) or chemokine receptors. Because binding of nickel ions to cysteine residues has already been demonstrated in nature, Ni-protein interaction may also happen via Cys residues in Erp44, despite the typical CXXC motif characteristic of oxidoreductases has been changed to CXXS in this molecule [118]. For Erp44, potential nickel histidine ligands exist between amino acids 350 to 370, but the exact binding ligand(s) remain(s) to be identified. Ni-protein interaction may alter Erp44 function, resulting in modified immune/allergic reactions by influencing the Ig production or the expression of Cys containing chemokine receptors, which co-regulate T cell homing.

From a more toxicological point of view molecular nickel has also been shown to interfere with gene silencing, DNA hypermethylation, the disturbance of DNA repair processes and the inhibition of histone acetylation [121,122]. If one of these observations is related to observed Ni-protein interaction with Nucleobindin 2, a calcium- and DNA-binding protein, or with heterotetrameric DNA-binding p52/p100 complex identified in our study, is a challenging question and requires further investigations [26,123].

Taken together, metalloproteomic analysis and identification of Ni-protein interactions in human B cells gives new insights into potential intracellular pathways involved in human nickel allergy or nickel metabolism, e.g. linking innate and adaptive immune responses via heat shock proteins or by interacting with danger molecules involved in potential Ni-induced toxic processes. Future studies such as comparative analyses of Ni-interacting or -regulated proteins of healthy donors and Ni allergic patients will help the basic knowledge of both metabolic and disease-related effects of Ni.

CONCLUSIONS

For a better understanding of the pathomechanisms of chemical-induced diseases like ACD, it will be essential to investigate the mechanisms by which chemicals, upon interaction with target proteins, activate the innate immune system to induce sensitization. A great effort has to be made to elucidate crucial parameters in this process such as factors which determine bioavailability, mechanisms of uptake of chemicals by cells and the processes of their metabolization and eventually elicitation thresholds. The combination of the increase in knowledge about the physiology and immune mechanisms of these diseases and the improvement of *in vitro* test systems for sensitizing potency and risk assessment, including the incorporation of genomics and proteomics, will help to avoid the use of harmful chemicals in consumer products. Furthermore, this knowledge will aid in the development of specific strategies for improved diagnostics and risk assessment for patients and to interfere with the interaction of chemicals with proteins to prevent immunopathology in AID or allergies.

ACKNOWLEDGEMENTS

We thank Dr. Ian D. Haidl, Dalhousie University, Dept. of Pediatrics and Microbiology and Immunology, Halifax, Canada, for careful reading of the manuscript.

This work was supported in part by the Landesstiftung Baden-Wuerttemberg, Germany, Forschungsprogramm "Allergologie" by grant P-LS-AL/26 to H.J.T.

ABBREVIATIONS

ACD	=	Allergic contact dermatitis
APC	=	Antigen presenting cell
CCT	=	Chaperonin-Containing TCP-1
CHS	=	Contact hypersensitivity
DC	=	dendritic cell
DNCB	=	2,4-Dinitrochlorobenzene
HSA	=	Human serum albumin

Hsp = Heat shock protein

LLNA = Local Lymph Node Assays

MAPK = MAP kinase

Ni-NTA = Nickel-Nitrilotriacetic Acid

PAMPS = Pathogen associated molecular patterns

PDI = Protein disulfide isomerase

SLs = Sesquiterpene lactones

TCP-1 = T-Complex Polypeptide-1

TLR = Toll-like receptor

TNCB = 2,4,6-Trinitrochlorobenzene

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Received: September 06, 2005 Revised: October 04, 2005 Accepted: October 07, 2005