



Research report

Novel fluorescence assay for tracking molecular and cellular allergen–protein interactions

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Abstract

T cells recognizing nickel (Ni) are key mediators in human Ni allergy, which represents the most common form of human contact hypersensitivity. In contrast to well-characterized Ni-specific human T cell clones, molecular knowledge about the extra- and intracellular route(s) of antigen/allergen presentation and processing of Ni-specific epitopes is still fragmentary. Here, we demonstrate a new metal-specific fluorescent technique to detect and quantify metal ions, like Ni²⁺, while they are associated with isolated metalloproteins. Moreover, utilizing the fluorescent metal sensor molecule Newport Green® (NPG) a novel method has been developed, which permits the metal-specific detection of Ni²⁺ binding to surface or intracellular structures of individual human antigen presenting cells by flow cytometry. We expect such metal-specific fluorescent analyses to contribute to a better basic understanding of molecular and cellular immune processes involved in Ni-specific T cell epitope generation and the pathogenesis of human nickel allergy.

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1. Introduction

Functional metal–protein interactions are essential in all living systems. Some metals may act as regulatory or

Abbreviations: DSA, dog serum albumin; HSA, human serum albumin; NPG, Newport Green®, fluorescent indicator for metals; NPG-Ac, Newport Green™; DCF diacetate, cell permeant; NPG-K, Newport Green™; DCF, dipotassium salt, cell impermeant.

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metabolic cofactors in enzymes while others play a fundamental role as structural elements in many well-characterized proteins (Finney and O'Halloran, 2003). Classical examples are P450-enzymes, hemoglobin, or integrin receptors in the immune system that require divalent cations for receptor function (Dransfield and Hogg, 1989). Regulation of ion homeostasis and cellular signalling is also controlled in part by metal–protein interactions, as exemplified by metal-specific ion channels (Dorovkov and Ryazanov, 2004; Prost et al., 2004).

Metal–protein interactions can also play a role in triggering pathological conditions, such as the most common form of human contact allergy, hypersensitivity to nickel. Activated metal-specific T cells are key mediators in Ni-specific allergic reactions, with metal-

specific T cell receptors triggered through transient metal-ligand-MHC molecule complexes on human antigen presenting cells (Vollmer et al., 1997; Gamberdinger et al., 2003; Lu et al., 2003). To uncover and specify the physiological and pathophysiological functions of metal ions like Ni^{2+} , Zn^{2+} and Cu^{2+} or Fe^{2+} it is obligatory to identify their corresponding protein targets (She et al., 2003; Heiss et al., 2005) as well as their binding domains and metal-controlled DNA regulatory elements (Murata et al., 1999; Huang, 2003; Hentze et al., 2004; Maxwell and Salnikow, 2004; Wang et al., 2004; Kindermann et al., 2005). However, it is also essential to monitor the binding of metal ions to cell membranes, the kinetics and routes of their cellular uptake, subcellular localization, and ion-induced metabolic pathways in cellular signalling.

Analytical techniques like graphite furnace atomic absorption spectrometry (GF-AAS) and inductively coupled plasma-mass spectrometry (ICP-MS) or X-ray fluorescence (XRF) spectrometry, allow efficient qualitative and quantitative detection of metals, e.g. of Ni^{2+} , Co^{2+} , Cu^{2+} , Fe^{2+} , Mn^{2+} or Zn^{2+} . However, these methods suffer from several disadvantages which preclude their use with physiologically active biological specimens. Thus, with these methods it is not possible to study the subcellular distribution or time-dependent binding or uptake of the metal ions quantified in a living cell. Fluorescent-based techniques are often able to overcome such limitations to permit the dynamic assessment of physiological processes in living systems. In fact, fluorescent probe-based measurements of intracellular Ca^{2+} and Zn^{2+} are commonplace (June and Rabinovitch, 1990; van Eeden et al., 1999; Meldolesi, 2004). However, similar assays for other metalloproteins and metal ions, such as Ni^{2+} , have not been described.

So far to elucidate unknown metal-specific processes and identify metallochaperones involved in human nickel allergy, we used metal-specific fluorescent sensor molecules to establish new metal-specific *in vitro* assays, including the quantitative analysis of metals interacting with isolated proteins (metalloproteins), and the detection and quantification of metal ion interaction with human immune cells in the fluorescence activated cell sorter (FACS).

2. Materials and Methods

2.1. Materials

$\text{NiSO}_4 \times 6\text{H}_2\text{O}$, $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, $\text{CoCl}_2 \times 6\text{H}_2\text{O}$, $\text{CuSO}_4 \times 5\text{H}_2\text{O}$, $\text{MnSO}_4 \times \text{H}_2\text{O}$, $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$ and serum albumin of human and dog origins were purchased

from Sigma (Deisenhofen, Germany) and stored in stock solutions at -20°C . $\text{MgCl}_2 \times 6\text{H}_2\text{O}$ was purchased from Baker, Deventer, NL, and $\text{Fe}_2(\text{SO}_4)_3 \times 5\text{H}_2\text{O}$ from Aldrich, Steinheim, Germany. His-tagged ubiquitin was from Dianova, Hamburg, Germany. Metal indicators Newport Green™ DCF diacetate (NPG-Ac, cell permeant) and Newport Green™ DCF, dipotassium salt (NPG-K, cell impermeant), were from Molecular Probes (Leiden, NL). Black 96 well fluoroassay microplates (polystyrene) were obtained from Costar, Corning B.V. Life Sciences, Schiphol-Rijk, NL. Allophycocyanin (APC) conjugated anti-human-CD19 (murine IgG1, clone HIB19) and murine APC-labeled IgG isotype controls were purchased from BD Biosciences, Heidelberg, Germany.

2.2. Human cells

Human Raji B cells were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Growth medium for B cells was RPMI 1640, supplemented with 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate and $1 \times$ mixture of nonessential amino acids (all from Gibco BRL, Eggenstein, Germany) and contained 10% heat-inactivated fetal calf serum (FCS). Human peripheral blood mononuclear cells (PBMC) were prepared by density gradient centrifugation (Ficoll, Amersham Biosciences, Freiburg, Germany) of heparinised venous blood. Isolated cells were cultured and immediately kept in growth medium (RPMI 1640) containing 10% fetal calf serum (Biochrom, Berlin, Germany).

2.3. Microplate-based fluorescent detection of metal–protein interactions

To generate metal–protein complexes, albumins of human (HSA, 300 μM) or dog serum (DSA, 300 μM) or His-tagged ubiquitin (110 μM) were incubated for 6 h at 37°C in 10 mM NiSO_4 and extensively dialyzed against phosphate buffered saline (PBS) or 0.9% NaCl at 4°C .

Solutions of different metals or *in vitro* generated metalloproteins were loaded into the microtiterplate wells (50 μl per well; added in concentrations as indicated), NPG-K was added (50 μl per well; 1 μM) and the plate was incubated at 25°C for 10 min before plates were measured in a SPECTRmax® Gemini XS plate reader (Molecular Devices, Sunnyvale, CA, USA) with excitation and emission wavelengths of 505 and 535 nm, respectively, and a cut-off filter of 530 nm. Measurements were usually performed in triplicate, and

data were fitted using a PRISM program (GraphPad Software, San Diego, USA).

2.4. Extracellular and intracellular metal detection by FACS analysis

The recently developed metal sensing fluorogenic dye Newport Green® (NPG) specifically recognizes ions such as Ni^{2+} , Zn^{2+} and Co^{2+} (Haugland, 2002). For the detection of cell surface bound Ni^{2+} cells were washed with 0.9% (w/v) NaCl and incubated for 1 h at 37 °C with various metal salts at indicated concentrations. Following extensive washing, cells were incubated for 30 min with 1 μM NPG-K and analyzed by FACS analysis (FACS Calibur, BD Biosciences; excitation 488 nm, emission filter 530/30 nm, FL-1), using CellQuest software (BD Biosciences).

When intracellular uptake of Ni^{2+} was measured, cells were incubated for 12 h at 37 °C, with metal salts at indicated concentrations, followed by 30 min incubation of cell permeant 1 μM NPG-Ac. If not indicated elsewhere, data represented were derived from at least three independent experiments.

3. Results and discussion

3.1. Microplate-based fluorescent detection of metal–protein interactions

To verify and standardize metal-specific fluorescent signals by Newport Green (NPG) a series of different cations was tested in a microplate-based fluorescent detection assay. As expected and clearly demonstrated in Fig. 1A, the metal-specific fluorescent sensor molecule NPG-K detects metal ions such as Ni^{2+} , Zn^{2+} , and Co^{2+} , whereas other divalent cations such as Fe^{2+} , Mn^{2+} , Ca^{2+} and Mg^{2+} were without effect. However, at concentrations of 0.5–5 μM Ni^{2+} induces significantly stronger fluorescence signals than Zn^{2+} , and Co^{2+} was even less effective (Fig. 1A). With ascending ion concentrations these differences qualitatively diminish, confirming recently published data (Haugland, 2002).

In biological systems metals are most often complexed to enzymes or to structural proteins, and therefore are not available in the free ionic state (Finney and O'Halloran, 2003). Prominent examples are hemoglobin, cytochrome C, or metal carriers like transferrin, plasma protein ceruloplasmin or human serum albumin (HSA). In the case of HSA, metal–protein interactions have been studied in more detail, with two well-characterized metal binding sites described for Cu^{2+} and Ni^{2+} ions. In contrast to dog serum albumin (DSA) most

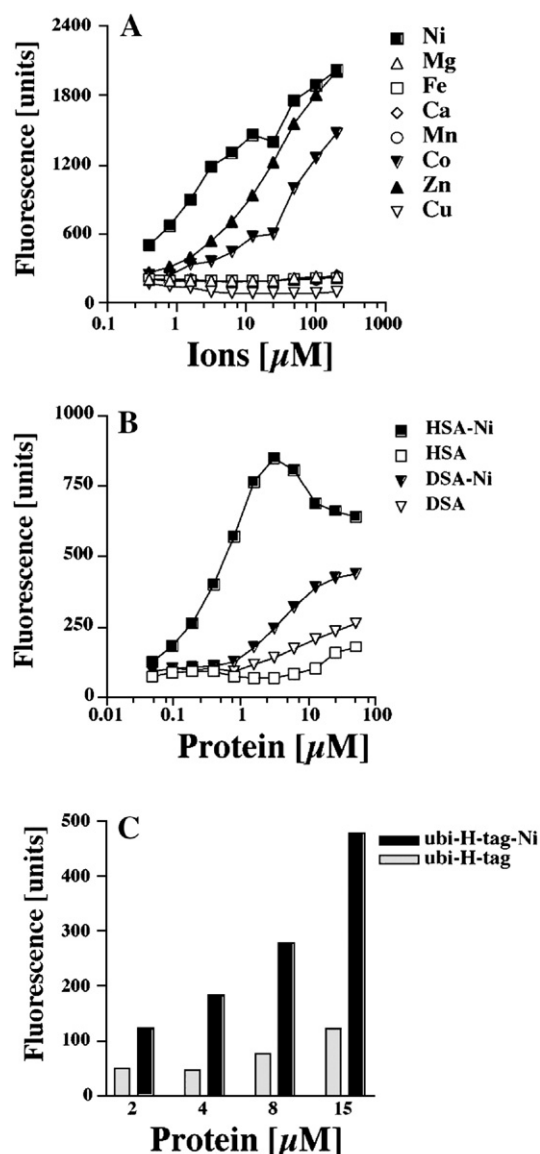


Fig. 1. Metal ion detection by NPG-K. (A) Fluorescence intensity of NPG-K (1 μM) as a function of graded concentrations of NiSO_4 , ZnSO_4 , CoCl_2 , CaCl_2 , CuSO_4 , MnSO_4 , MgCl_2 or $\text{Fe}_2(\text{SO}_4)_3$. (B) NPG-K fluorescence as a function of the concentration of human (HSA) or dog serum albumin (DSA) or the respective NiSO_4 treated proteins HSA-Ni and DSA-Ni. (C) NPG-K fluorescence in the presence of recombinant His-tagged ubiquitin, treated or untreated with NiSO_4 . Excitation wavelength was 505 nm and emission wavelength was 535 nm.

mammalian albumins bind both Cu^{2+} and Ni^{2+} more tightly and more specifically than they bind other cations. Metal–albumin interactions involve the amino terminus, with an obligate histidine in position three, which is absent in DSA (Glennon and Sarkar, 1982; Mohanakrishnan et al., 1985).

We have recently demonstrated that metalloproteins such as HSA-Ni (Ni^{2+} complexed to HSA), may have an important bifunctional role in human nickel allergy: first, as a potential metal-trafficking protein in human skin, and secondly as a functional molecule in Ni-specific human T cell clone activation (Thierse et al., 2004; Thierse et al., 2005; Martin et al., 2006). In this study we examined the principal capacity of the metal indicator NPG-K to detect Ni^{2+} in a protein-bound state. As clearly shown in Fig. 1B, NPG-K also allows specific detection of Ni^{2+} ions when complexed to HSA, implying that such *in vitro* generated Ni^{2+} -protein-complexes still maintain a binding potential for NPG.

In addition, the comparison of Figs. 1A and B reveals that both 1 μM HSA-Ni and 1 μM NiSO_4 induced equivalent NPG-K fluorescent signals, with 690 fluorescence units for 1 μM HSA-Ni and 660 fluorescence units for 1 μM NiSO_4 , respectively. This is in accordance with our previously published data (Thierse et al., 2004) demonstrating an approximately 1:1 molar ratio of Ni to protein in HSA-Ni. The data in Fig. 1B further corroborate the previous finding (Thierse et al., 2004) that dog serum albumin (DSA), lacking the Ni-interacting His at position three, is an extremely poor Ni-binder.

Fig. 1C depicts binding of Ni^{2+} to His-tagged recombinant ubiquitin as detected by NPG-K fluorescence. Apart from representing an additional example for the detection of protein-bound Ni^{2+} by NPG, these data reveal that His-tagged recombinant proteins, upon purification based on Ni affinity, may turn out as unexpected carriers of Ni^{2+} . Since the bound Ni^{2+} may interfere with functional assays of such proteins, a simple purity control of His-tagged proteins isolated by Ni^{2+} -based technology with the metal sensing NPG assay could be of great importance (Garzia et al., 2003; Sun et al., 2005). The microplate-based metal-specific fluorescence assay as presented here provides a versatile new method to detect metal ions like Ni^{2+} in physiological important metal transporters. This may be applicable to the monitoring of Ni^{2+} -ion-transfers between different proteins in common metal-related diseases. Two such examples are *Helicobacter pylori* infections, with Ni-requiring urease essential for stomach colonization, or human nickel allergy, mediated by Ni-specific human T cells (Kapsenberg et al., 1988; Thierse et al., 2004; van Vliet et al., 2004; Ledwidge et al., 2005).

3.2. Extracellular and intracellular metal detection by FACS

To detect metal ions bound extracellularly to membranes of living cells, we examined the capacity, speci-

ficity, and efficiency of NPG-K, which cannot permeate cellular membranes. Antigen presenting cells are obligatory in T cell-mediated human nickel allergy. Human B cells (Raji and CD19 positive PBMC), which may act as such metal-epitope presenting cells, were chosen and incubated with equal concentrations of Ni^{2+} , Zn^{2+} and Co^{2+} (10 μM). After subsequent addition of NPG-K, cells were analyzed by FACS and compared to cells incubated without metal salt solutions. As depicted in Fig. 2A, D and F, the fluorescence signal increased significantly when 10 μM Ni^{2+} was added, but only barely in the presence of 10 μM Zn^{2+} , and not at all of 10 μM Co^{2+} , indicating a rather specific signal for Ni^{2+} ions. Notably, even in the absence of supplementary metal ions a high background signal of NPG-K fluorescence occurred, possibly due to constitutive metal-membrane receptor interactions or unspecific binding of NPG-K to the outer cell surface. To further evaluate a potential metal concentration-dependent NPG-K fluorescence signal, increasing concentrations of Ni^{2+} were added to human B cells (Fig. 2B, C) followed by metal sensor (NPG-K) detection. Resulting mean fluorescence intensities indicated a small dynamic range for the detection of Ni^{2+} by NPG-K between 1 μM and 100 μM Ni^{2+} (Fig. 2C). Concentrations above 250 μM Ni^{2+} did not lead to a further increased NPG-K signal. Incubations with higher concentrations of Zn^{2+} (500 μM , Fig. 2E) did not increase the fluorescence signal as compared to 10 μM Zn^{2+} (Fig. 2D). Thus, although Zn^{2+} and Co^{2+} are capable of binding to NPG-K (Fig. 1A), their surface-associated concentrations on Raji cells, in contrast to Ni^{2+} , are too low to be detected by NPG-K fluorescence (Haugland, 2002).

These data reveal that cellular surface binding of Ni^{2+} induces a metal-specific, NPG-K mediated fluorescence signal, which is detectable on a single cell level by standard FACS technology. The new method might be particularly advantageous in analyzing and tracking metal ions bound to carrier molecules. These carriers may then be subsequently delivered to cellular receptors of human antigen presenting cells and T cells, thus mediating Ni-specific human T cell activation in human Ni allergy (Thierse et al., 2004; Weltzien et al., 2006).

To determine intracellular uptake of Ni^{2+} by human B cells, we employed the cell permeant fluorescent ion sensor NPG-Ac. NPG-Ac was added to the cells after overnight incubation with Ni^{2+} . Upon penetration through the cellular membrane NPG-Ac is deacylated by endogenous esterases. This traps the molecule within the cell and at the same time activates its ion sensor structure. Similar to studies by others, who successfully applied NPG-Ac to determine intracellular Zn^{2+} in neurons and pancreatic

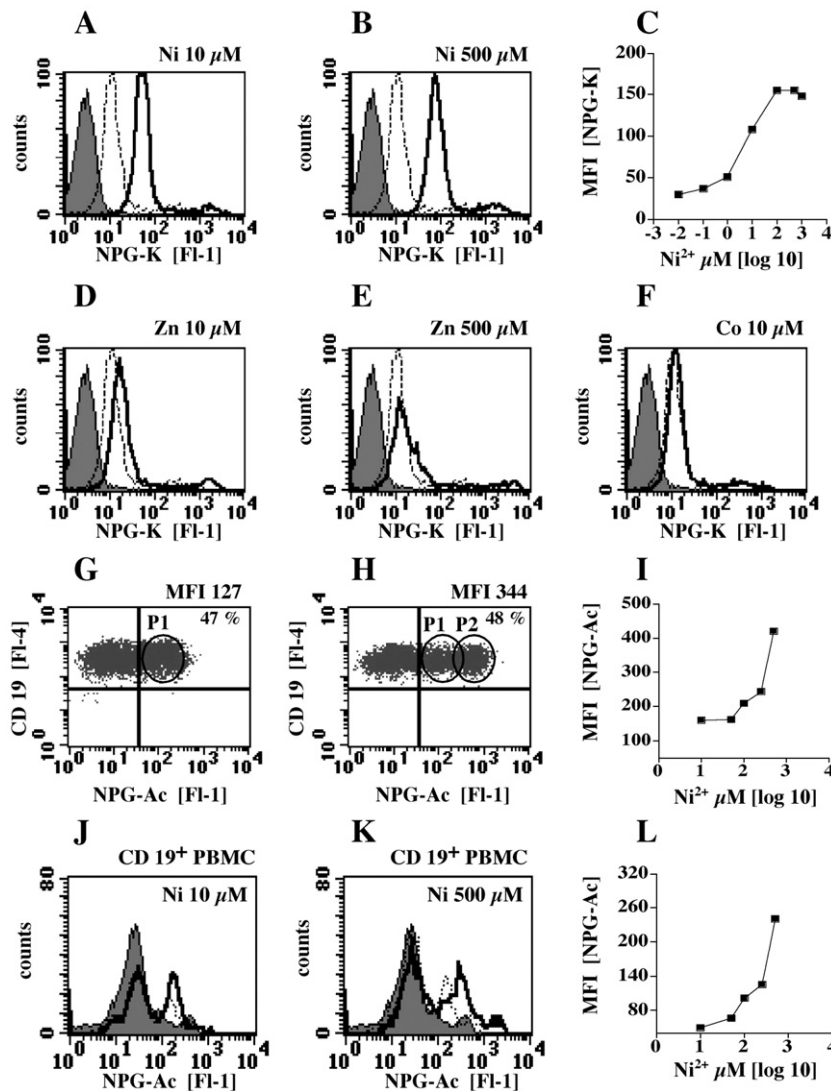


Fig. 2. NPG-K specifically recognizes Ni²⁺ on human Raji B cell surface membranes. Raji cells were washed with 0.9% (w/v) NaCl and incubated for 1 h at 37 °C with increasing concentrations of NiSO₄ (A–C), ZnSO₄ (D, E) or CoCl₂ (F). Following extensive washing, cells were incubated for 30 min with NPG-K (1 μ M) and analyzed by FACS. Histograms show controls in the absence of NPG-K (filled), and NPG-fluorescence before (dotted lines) and after treatment with metal salts (continuous lines). To analyze intracellular Ni²⁺ uptake, human CD19 positive Raji B cells were incubated for 12 h with Ni²⁺ and subsequently with cell permeable NPG-Ac (1 μ M); signals from endogenous esterase activated and intracellular trapped NPG-Ac were analyzed by FACS (G, without Ni²⁺; H, with 250 μ M Ni²⁺, or other Ni²⁺ concentrations shown as the mean fluorescence intensity (MFI) of NPG-Ac as in I). Human B cell surfaces were stained with anti-CD19 antibody (IgG1-APC, clone HIB 19) as described in Materials and methods. Esterase-dependent catalyzed NPG-Ac recognizes Ni²⁺ in freshly isolated human B cells: human PBMC were incubated overnight (12 h) at 37 °C with increasing concentrations of NiSO₄ (J–L). Following extensive washing, cells were incubated for 30 min with NPG-Ac (1 μ M), gated for CD19⁺ B cells to distinguish them from other NPG-Ac positive cells, and signals from endogenous esterase activated and intracellular trapped NPG-Ac were analyzed by FACS. Histograms (J,K) show controls in the absence of NPG-Ac (filled) and in the presence of NPG-Ac (dotted lines), and after treatment with metal salts (continuous lines). Human B cell surfaces were stained with anti-CD19 antibody (IgG1-APC, clone HIB 19).

cells (Lukowiak et al., 2001; Wei et al., 2004), we were able to detect intracellular Ni²⁺ by FACS (Fig. 2). These analyses demonstrate a high background signal for NPG-Ac alone (Fig. 2G, 47% cells positive, MFI in P1: 127),

possibly due to dye-specific characteristics or other intracellularly distributed metal ions. However, when the cells were pre-incubated with 250 μ M Ni²⁺, the signal increased significantly (Fig. 2H; MFI in P2: 344). The

intracellular fluorescent signal increased further with increasing concentrations of Ni^{2+} as shown in Fig. 2I (linear range from 10 to 500 μM Ni^{2+} , MFI 180 to 415), but differed from those obtained for extracellular Ni^{2+} with NPG-K (Fig. 2C; linear range from 1 to 100 μM Ni^{2+} , MFI 50 to 170). The discrete populations in Fig. 2G and H indicate i) a differential uptake of NPG-Ac into human Raji cells and ii) a non-homogeneous distribution of intracellular Ni^{2+} .

Very similar results were seen for B cells in freshly isolated human PBMC (Fig. 2J–L). More specifically, we analyzed the $\text{CD}19^+$ sub-population within NPG-Ac treated PBMC (FACS gating for $\text{CD}19^+$ cells). As indicated in Fig. 2J, this population contained a significant sub-population exhibiting a NPG-Ac specific fluorescence (dotted line) and no significant signal when pre-incubated with low Ni concentrations (continuous line), similar to Raji cells. Pre-incubation with increasing Ni concentrations here also resulted in a third population of strong fluorescence, comparing well to signals mediated by NPG-Ac in human Raji cells (Fig. 2K and L). Moreover, the simultaneous addition of 100 μM NiSO_4 and 30 μM HSA resulted in a 42% (significant) reduction of NPG-Ac fluorescence (data not shown). This is in contrast to data for the non-penetrating NPG-K (Fig. 1B) where free or HSA-bound Ni^{2+} apparently result in comparable fluorescence signals.

4. Conclusion

Lacking appropriate fluorescent assays to monitor metal allergens such as Ni^{2+} complexed with proteins or associated with human antigen presenting cells, new metal-specific fluorescent assays have been developed. First, a simple microtiter-based fluorometric assay has been established to reproducibly quantify not only metal ions in aqueous solution such as Ni^{2+} in $\text{NiSO}_4 \times 6\text{H}_2\text{O}$, Co^{2+} in $\text{CoCl}_2 \times 6\text{H}_2\text{O}$, and Zn^{2+} in $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$, but also metal ions complexed in metalloproteins. Second, a novel FACS method was developed to determine metal ions associated extracellularly or intracellularly with living human antigen presenting cells. Thus, combining established Ni^{2+} -specific T cell activation assays with metal-specific fluorescent detection assays should permit new insights into molecular processes involved in the pathophysiology of human nickel allergy.

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