

Metal-Protein Complex-Mediated Transport and Delivery of Ni²⁺ to TCR/MHC Contact Sites in Nickel-Specific Human T Cell Activation¹

Hermann-Josef Thierse,^{2*} Corinne Moulon,^{3*} Yvonne Allespach,^{4*} Bastian Zimmermann,[†] Andrea Doetze,^{*} Stephan Kuppig,^{*} Doris Wild,^{*} Friedrich Herberg,[‡] and Hans Ulrich Weltzien^{*}

Nickel allergy clearly involves the activation of HLA-restricted, skin-homing, Ni-specific T cells by professional APCs. Nevertheless, knowledge concerning the molecular details of metal-protein interactions underlying the transport and delivery of metal ions to APC during the early sensitization phase and their interactions with HLA and TCRs is still fragmentary. This study investigates the role of human serum albumin (HSA), a known shuttling molecule for Ni²⁺ and an often-disregarded, major component of skin, in these processes. We show that Ni-saturated HSA complexes (HSA-Ni) induce and activate Ni-specific human T cells as potently as Ni salt solutions when present at equimolar concentrations classically used for in vitro T cell stimulation. However, neither HSA itself nor its Ni-binding N-terminal peptide are involved in determining the specificity of antigenic determinants. In fact, HSA could be replaced by xenogeneic albumins exhibiting sufficient affinity for Ni²⁺ as determined by surface plasmon resonance (Biacore technology) or atomic absorption spectroscopy. Moreover, despite rapid internalization of HSA-Ni by APC, it was not processed into HLA-associated epitopes recognizable by Ni-specific T cells. In contrast, the presence of HSA-Ni in the vicinity of transient contacts between TCR and APC-exposed HLA molecules appeared to facilitate a specific transfer of Ni²⁺ from HSA to high-affinity coordination sites created at the TCR/HLA-interface. *The Journal of Immunology*, 2004, 172: 1926–1934.

Contact allergy to nickel (Ni), the most common form of allergic contact dermatitis, is a T cell-controlled disease (1–3). Ni-specific, IFN- γ -producing CD4⁺ and CD8⁺ effector T cells are believed to be responsible for sensitization as well as for skin reactions (4, 5), whereas IL-10- and TGF- β -secreting CD4⁺ regulatory T cells are thought to down-modulate the response (2). Ag-specific activation of these cells upon skin contact with Ni-containing alloys such as the new one- and two-euro coins requires translocation of Ni²⁺ from the surface to Langerhans cells (LC)⁵ in deeper layers of the skin (6, 7). According to current models, LC will thereby be stimulated to mature and migrate to local lymph nodes where they supposedly present Ni-HLA

epitopes to T cells (7, 8). Rechallenge with Ni initiates the effector phase of allergic contact dermatitis, resulting in characteristic skin lesions. The clinical symptoms are accompanied by rapid infiltration of allergen-specific, cutaneous lymphocyte-associated Ag- and CCR4-positive, T cells into dermis and epidermis at the site of Ni application (3, 9). The recruited effector T cells appear to be activated on the spot and not to require LC migration.

Neither the molecular nature of Ni-induced antigenic determinants nor the very early molecular events of metal transport through the human epidermis to LC have yet been satisfactorily resolved. The necessity of Ni transport may be indicated by the fact that Ni-reactive T cells, and hence probably Ni²⁺ as well, persist in skin in the vicinity of the sensitizing Ni application for a remarkably long time (10). Thus, Ni²⁺ appear to be captured and kept in place by complexation, e.g., to histidine-rich proteins or their metabolic breakdown products such as filaggrin in the outer cornified layers of skin (11, 12). Any translocation toward deeper epidermal layers might, therefore, require a transfer of Ni²⁺ to mobile carrier proteins or peptides.

One classical Ni-interacting protein is human serum albumin (HSA) (13–15). HSA is the most prominent plasma protein and represents a prototypic carrier molecule for a wide variety of molecules including fatty acids, lipids, vitamins, or various metals and drugs (14). For many of these interacting molecules, albumin provides a depot so they will be available in concentrations well above their solubility in plasma. In other cases, it removes toxins from the circulation and transports them to disposal sites. In addition, albumin may turn itself into a regulatory protein-ligand complex by stabilizing otherwise short-lived biological functions of the ligand. One example is the formation of S-nitroso-albumin by reaction with NO. S-Nitroso-albumin retains the vasoactive properties of NO at 1000-fold-extended half-life (16–18).

*Max-Planck-Institut für Immunbiologie, Freiburg, Germany; [†]BIAFFIN GmbH & Co KG, and [‡]Abteilung Biochemie, Universität Kassel, Kassel, Germany

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² Address correspondence and reprint requests to Dr. Hermann-Josef Thierse, Max-Planck-Institut für Immunbiologie, Stübeweg 51, D-79108 Freiburg, Germany. E-mail address: thierse@immunbio.mpg.de

³ Current address: Dictagene, Chemin de la Vulliette 4, CH-1000 Lausanne 25, Switzerland.

⁴ Current address: CellGenix Technologie Transfer GmbH, Am Flughafen 16, D-79108 Freiburg, Germany.

⁵ Abbreviations used in this paper: LC, Langerhans cell; HSA, human serum albumin; CSA, chicken serum albumin; DSA, dog serum albumin; PSA, pig serum albumin; MSA, mouse serum albumin; HSA-Ni, in vitro-generated HSA-nickel metalloprotein complex; HS, human serum; DC, dendritic cell; mDC, mature DC; iDC, immature DC; NPG, Newport Green; h, human; m, mouse; SPR, surface plasmon resonance; NTA, nitrilotriacetic acid; TNP, trinitrophenyl.

For Ni and other heavy metals, the major role of HSA may be that of a physiological detoxifier, facilitating metal removal via the kidney. However, HSA may also deliver Ni^{2+} to APC such as LC in the skin. In this context, it is of interest that HSA is particularly abundant in skin, the largest immunological organ (14). As a transporter of essential nutrients for epithelial cells in the absence of blood vessels, HSA efficiently crosses the epidermal basement membrane (19). Moreover, the dynamic variation of HSA concentrations in relation to skin hydration may indicate that HSA may also cross the basement membrane in the opposite direction. Therefore, HSA might well be a prime candidate to shuttle Ni^{2+} to APC in lower layers of the skin.

Previous studies from our laboratory have already shown that human Ni-reactive T cell clones could indeed be induced by Ni^{2+} complexed to HSA (HSA-Ni) (5, 20). Subsequently, similar data have been reported by Artik et al. (21) for murine T cells. The present study presents evidence that HSA-Ni serves as an intermediate interaction partner for T cell stimulation by Ni^{2+} and that the HSA protein itself is not involved in determining epitope specificity. We also show by surface plasmon resonance (SPR; Biacore) and Ni transfer experiments that the affinity of HSA for Ni^{2+} is low enough to allow for effective transfer of Ni^{2+} to coordination sites of equal or higher affinity on other proteins or peptides.

Materials and Methods

Media and reagents

Growth medium for T cell clones (RPMI-human serum (HS)) was RPMI 1640, supplemented with 2 mM L-glutamine, 5×10^{-5} M 2-ME, 1 mM sodium pyruvate, and 1× mixture of nonessential amino acids (all from Life Technologies, Eggenstein, Germany) and 5% pooled human AB serum (Red Cross Transfusion Center, Basel, Switzerland). Medium for hybridoma and other cell lines (RPMI-FCS) contained 10% heat-inactivated FCS. X-VIVO-15 (BioWhittaker, Verviers, Belgium), supplemented with 1% heat-inactivated autologous plasma, was used for *in vitro* generation of dendritic cells (DC). Recombinant human cytokines rIL-4, rIL-6, and rIL-1 β were from PeproTech (Rocky Hill, NJ), rTNF- α was from Strathmann (Hannover, Germany), GM-CSF was from Novartis (Basel, Switzerland), and PGE₂ was from Pharmacia (Erlangen, Germany). Serum albumin of human (with or without FITC labeling), bovine, murine, chicken, dog, and porcine origin, NiSO₄ × 6H₂O, and NiCl₂ × 6H₂O were purchased from Sigma-Aldrich (Deisenhofen, Germany) and stored in stock solutions at -20°C. Peptides were synthesized by Bachem (Weil, Germany). Ni-detecting Newport Green (NPG) was from Molecular Probes (Leiden, The Netherlands).

Albumin-nickel complexes

Albumins of different species (HSA, HSA-FITC, BSA, chicken serum albumin (CSA), dog serum albumin (DSA), and pig serum albumin (PSA), 20 mg/ml; mouse serum albumin (MSA), 2 mg/ml) were incubated for 6 h at 37°C in 1 mM NiSO₄ and extensively dialyzed against PBS at 4°C. The Ni content of some of the preparations was analyzed by graphite furnace atomic absorption spectroscopy (Bioscientia, Ingelheim, Germany). Protein concentrations were determined using the Pierce BCA protein determination kit (Pierce, Rockford, IL).

T cells and cell lines

Ni-reactive T cell clones were obtained according to published procedures (5). They were cultured in RPMI-HS with 1 $\mu\text{g}/\text{ml}$ PHA-P (Murex Diagnostics, Dartford, U.K.) and 100 IU/ml recombinant human IL-2 (Proleukin; EuroCetus, Ratingen, Germany) on irradiated, allogeneic PBMC. Clones with prefix ANi (synonymous with HSA.Ni) from donor IF have been described before (5) as well as clone SE9 of donor SE (22). Clones of donor KG with prefix BC or SDC were newly prepared from PBMC or skin lesions, respectively.

The T cell hybridoma T23, obtained by expression of the TCR of clone ANi 2.3 in the hybridoma line 54 ζ 17 (23), has been characterized before (22, 24). The B cell line WT47, homozygous for *HLA-DRI3/DR52c*, was from the International Histocompatibility Workshop (no. 9063; Turin, Italy), and Raji cells were obtained from American Type Culture Collection (Manassas, VA). EBV transformation of donor B cells was performed as described (25).

Generation of DC

Human mature DC (mDC) and immature DC (iDC) were obtained by slight modification of published procedures (26, 27), using the serum-free X-VIVO-15 medium. Briefly, CD14⁺ monocytes were positively selected from Ficoll-purified PBMC of healthy donors by high-speed magnetic cell sorting (autoMACS; Miltenyi Biotech, Bergisch Gladbach, Germany). Sorted cells (3×10^6) were cultured for 7 days in 3 ml of X-VIVO-15 medium containing 1% autologous plasma, 800 U/ml human rGM-CSF, and 1000 U/ml rIL-4. Every other day, 1 ml of supernatant was replaced by fresh medium containing 1600 U/ml GM-CSF and 1000 U/ml IL-4. On day 7, cells were characterized by FACS staining as iDC, being negative for CD14 and CD83, low for CD58, CD80, and CD86, and high for CD40 and HLA-DR. For full maturation, cells were washed on day 7, and 1×10^6 cells were transferred into six-well plates in 3 ml of X-VIVO-15 medium containing 800 U/ml GM-CSF and 1000 U/ml IL-4, and stimulated with 10 ng/ml TNF- α and IL-1 β , 1000 U/ml IL-6, and 1 $\mu\text{g}/\text{ml}$ PGE₂. After an additional 2 days, cultures contained typical mDC, phenotyped as CD14 negative, CD83 positive, and high for CD58, CD80, CD86, CD40, and HLA-DR.

T cell activation assays

Ni-specific proliferation of T cell clones (2×10^4) was determined in the presence of graded concentrations of NiSO₄, NiCl₂, or albumin-Ni complexes on 2×10^4 irradiated autologous PBMC (3000 rad) or autologous EBV-transformed B cell lines (EBV-B cells) (6000 rad) as Ag APC in 200 μl of RPMI-HS. After 48 h, cultures were pulsed for 18 h with 2 $\mu\text{Ci}/\text{well}$ [³H]thymidine, and radioactivity uptake into the DNA was determined on GF/A filters in an automatic beta counter (Inotech, Asbach, Germany). Hybridoma cells T23 (5×10^4 cells/well) were cultured for 20 h with Ag on 5×10^4 irradiated APC in 200 μl of RPMI 1640 medium with or without 10% FCS. Culture supernatants were used for IL-2 determination using an IL-2-dependent CTLL line as described previously (28). Briefly, IL-2-dependent CTLL proliferation was measured after 24 h by an 18-h pulse with [³H]thymidine as above. To examine processing-independent T cell activation, APC were fixed for 45 s with 0.05% glutaraldehyde (29). In Ag-pulsing experiments, APC were treated for 1 h at 37°C with HSA-Ni (1 mg/ml) or NiSO₄ (1 mM) and washed extensively before being used in stimulation assays.

Flow cytometry and Abs

Direct or indirect immunofluorescence stainings were performed on ice in a PBS buffer containing 3% FCS. For staining with HSA-FITC, cells were incubated with varying amounts of HSA-FITC at different temperatures and for different times. In some experiments, cells were counterstained with anti-CD19-PE mAb or anti-CD40-PE. Cells were then washed and fixed in 2% paraformaldehyde. Analyses were conducted in a FACScan instrument using CellQuest software (BD Biosciences, Mountain View, CA).

Abs used were as follows: human (h)CD19-PE (mouse (m)IgG1; clone HIB19; BD Biosciences), hCD40-PE (IgG1; clone mAb89; Immunotech, Luminy, France), hCD14-PE (clone M5E2; BD PharMingen, San Diego, CA), hCD80-FITC or -PE (clone MAB104; Immunotech), hCD83-FITC (clone HB15e; BD PharMingen), hCD86-FITC (clone B463; Biozol, Eching, Germany), HLA-DR-FITC (clone L243; BD PharMingen), and hCD58-FITC (clone AICD58; Immunotech). Murine PE- or FITC-labeled IgG isotype controls were from Sigma-Aldrich.

Fluorometric detection of cellular bound Ni²⁺

Fluorogenic NPG (Molecular Probes) specifically recognizes Zn²⁺ and Ni²⁺ (30). For detection of cell-bound Ni²⁺, cells were washed with 0.9% (w/v) NaCl and incubated with Ni, Cu, albumin, and the metalloprotein HSA-Ni in concentrations as indicated, for 1 h at 37°C. Following extensive washing (or without), cells were incubated for 30 min with NPG (1 μM) and analyzed by FACScan, using CellQuest software (BD Biosciences).

Surface plasmon resonance

SPR experiments were performed using Biacore 2000 and 3000 instruments (Biacore, Uppsala, Sweden). The interaction between an immobilized component referred to as the ligand (Ni²⁺) and a molecule in the mobile phase, the analyte (e.g., HSA), was determined. Changes in surface concentration are proportional to changes in the refractive index on the surface resulting in changes in the SPR signal, plotted as resonance units. A value of 1000 resonance U corresponds to a surface concentration of 1 ng/mm² (31). For a review of the SPR technique, see Zimmermann et al. (32).

Table I. Metal-specific induction and activation of human T cell clones by HSA-Ni and NiSO₄

Donor	Clone	Induction	Stimulation Index ^a	
			HSA-Ni	NiSO ₄
IF	ANi 2.0	HSA-Ni	5.9	6.8
	ANi 2.2	HSA-Ni	11.4	15.0
	ANi 2.3	HSA-Ni	4.1	8.7
	ANi 2.4	HSA-Ni	7.3	10.6
	ANi 3.3	HSA-Ni	3.9	4.6
KG	BC.T 1	HSA-Ni	39	32
	BC.T 13	HSA-Ni	16	20
	BC.T 17	HSA-Ni	19	48
	SDC.S3	NiSO ₄	11	11
	SDC.S9	NiSO ₄	26	41
	SDC.S10	NiSO ₄	2	12
	SDC.S16	NiSO ₄	3	6
	SDC.S17	NiSO ₄	2	7
SE	SE 9	NiSO ₄	16	21

^a Proliferative responses of human T cell clones to either HSA-Ni (1 mg/ml) or NiSO₄ (10⁻⁴ M) in the presence of autologous EBV-B cell lines were assayed by incorporation of [³H]thymidine. Stimulation index refers to ratio of incorporation in the presence over that in the absence of Ag. Only data were used where the Ni-induced responses corresponded at minimum to 1000 cpm and where SDs were <20% for triplicates. Controls in the absence of Ag ranged from 200 to 2000 cpm. Ni-specific T cell clones of donors IF and SE have been already described (5, 22). Clones of donor KG were derived from either freshly isolated PBMC (BC) or Ni-induced skin lesions (SDC). Induction indicates whether primary in vitro stimulation was performed with NiSO₄ or HSA-Ni.

Sensor chip nitrilotriacetic acid (NTA) (NTA covalently immobilized on a carboxymethylated dextran matrix) and surfactant P20 were obtained from Biacore. All other reagents were obtained in the purest grade available. A Ni-NTA chip was inserted into the machine, and the machine was primed with both pumps in running buffer (10 mM HEPES, 150 mM NaCl, 50 μM EDTA, 0.005% p20 (pH 7.4)). Specific surfaces were treated with 500 μM NiCl₂ in running buffer. Proteins were also diluted into running buffer ranging from 20 nM to 40 μM or as indicated on the plots. All runs were performed at 20°C at a flow rate of 30 μl/min. Unspecific binding was subtracted using blank runs on a surface not loaded with Ni²⁺. The association rate was monitored for 300 s and the dissociation phase for 300 s. After each interaction, the surface was regenerated with subsequent injections of 3 M guanidinium hydrochloride in water and 350 mM EDTA in running buffer.

Because most interactions were mass transfer, limited kinetic data were not extracted unless steady-state analysis was possible. Otherwise, data are plotted as maximum binding at the end of the injection phase.

Confocal microscopy

For fluorescent detection of HSA-FITC and HSA-Ni-FITC by confocal microscopy, cells were incubated for 2 h in RPMI 1640 medium containing 500 μg/ml HSA-Ni-FITC. Membranes were counterstained with biotin-labeled anti-CD19 Abs (BD Biosciences) for 30 min, at 4°C, and in the

dark, followed by Texas Red-streptavidin (Life Technologies) for 30 min. After washing, cells were fixed in 2% paraformaldehyde (15 min; 4°C; dark), and embedded carefully in Fluoromont-G (Southern Biotechnology Associates, Birmingham, AL). Cells were imaged using a confocal microscope TCS SP2 UV system with spectral scanhead (Leica Microsystems, Mannheim, Germany).

Statistical analysis

Results are expressed as means ± SD. Differences between groups were assessed by the Student's *t* test. Values of *p* < 0.05 were considered to be statistically significant, and *p* value differences were defined by symbols: NS, >0.05; *, 0.01–0.05, significant; **, 0.001–0.01, very significant; and ***, <0.001, extremely significant.

Results

HSA-Ni complexes activate Ni-specific human T cells

Ni-saturated HSA (HSA-Ni) was produced by incubation of HSA with NiSO₄ and subsequent extensive dialysis. HSA-Ni complexes as well as free NiSO₄ were used in vitro to stimulate MHC class II-restricted, Ni-reactive human T cells from PBMC of Ni-allergic donors. Ni-reactive T cell cultures were cloned by limiting dilution, and clones were assayed for proliferative responses to either free NiSO₄ or HSA-Ni in the presence of irradiated, autologous, EBV-transformed B cells as APC. As shown by stimulation indices in Table I, the HSA-Ni-induced T cell clones proliferated quite comparably in response to HSA-Ni or NiSO₄, an observation confirmed for >25 individual clones (not all shown). In contrast, a notable proportion of the clones induced by NiSO₄ revealed lower to negligible responses to HSA-Ni as compared with the inducing NiSO₄.

A molar ratio of Ni to protein of 1.2 was determined in our HSA-Ni preparation by atomic absorption spectroscopy (Table II). This permitted the quantitative comparison of the activating potential of HSA-Ni and free Ni salts. As demonstrated in Fig. 1, the molar amount of Ni necessary for half-maximal proliferation of the HLA-DR-restricted clone SE9 was surprisingly independent of whether Ni²⁺ was added complexed to HSA or free in salt solution, indicating efficient transfer of ions from the metalloprotein to HLA-TCR contact regions.

Serum albumin as a mediator of Ni-specific T cell activation

The above findings and the fact that all of our experiments were performed in the presence of either human or bovine serum, suggested that Ni²⁺ in our medium might largely be bound to either HSA or BSA. To study this and other questions in more detail, we made use of the previously described transfectant T23, which expresses the Ni-reactive TCR of the human T cell clone ANi 2.3 in a murine hybridoma (24, 28). Like ANi 2.3 (Table I), T23 responded not only to NiSO₄ but also to HSA-Ni when presented by

Table II. Ni-binding capacity of xenogenic serum albumins determines their stimulatory potency for Ni-specific T cells^a

Albumin Complex	SWISS-PROT	N-Terminal Sequence	Molar Ratio Ni/Protein	Stimulation Index of Ni-Reactive Human T Cell Clones				
				ANi 2.0	ANi 2.2	ANi 2.3	ANi 2.4	ANi 3.3
HSA-Ni	P 02768	D A H K S E V	1.2	16.2	32.0	4.1	7.3	16.0
DSA-Ni	P 49822	E A Y K S E I	<0.01	1.1	2.7	1.2	1.6	1.1
BSA-Ni	P 02769	D T H K S E I	ND	6.6	8.5	3.7	4.8	3.1
CSA-Ni	P 19121	D A E H K S E	0.2	6.5	18.6	3.3	4.7	8.7
PSA-Ni	P 08835	D T Y K S E I	0.3	9.3	25.1	ND	ND	10.0
MSA-Ni	P 07724	E A H K S E I	ND	7.6	25.5	2.8	4.3	11.8

^a Ni-specific human T cell clones were activated by in vitro-generated syngeneic (HSA-Ni) and xenogenic metalloalbumins DSA-Ni, BSA-Ni, CSA-Ni, PSA-Ni, MSA-Ni, each at 1 mg/ml. To compare their Ni-binding capacities, albumins of different species were incubated with NiSO₄, and subsequently dialyzed against PBS. The molar ratios of Ni/protein were calculated from atomic absorption spectroscopy and protein determination by a BCA assay. T cell reactivity of clones to syngeneic and xenogenic metalloalbumins was measured by incorporation of [³H]thymidine, and results are expressed as stimulation indices (see Table I). Indices >3 are in bold. Sequences were derived from the SWISS-PROT database (<http://www.ebi.ac.uk>).

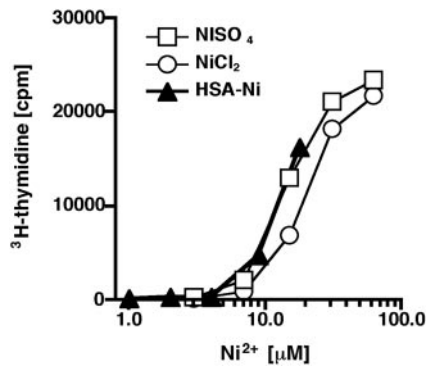


FIGURE 1. HSA-Ni induces Ni-specific T cell activation at equimolar concentrations when compared with NiSO_4 or NiCl_2 . The Ni-specific human T cell clone SE9 and APC were incubated with increasing equimolar Ni concentrations of HSA-Ni and NiSO_4 or NiCl_2 for 48 h, and T cell reactivity was assayed by incorporation of [^3H]thymidine after a further 18 h.

APC expressing the restricting human *HLA-DR52c* allele. Interestingly, we found that the presence of FCS enhanced T23 activation by NiSO_4 , but decreased the effectiveness of HSA-Ni (Fig. 2A). Hence, it appeared that the metalloprotein complex of HSA-Ni played an active and necessary role in the stimulation of Ni-specific T cells. This interpretation is in line with the finding (Fig. 2A) that dilution of HSA-Ni with Ni-free serum diminished its stimulatory effectiveness. One interpretation of these findings is that HSA-Ni facilitates the uptake of Ni by the APC. Following intracellular processing, Ni complexed to the N-terminal HSA peptide might then be presented on the APC's MHC molecules. However, serum albumin or the N-terminal Ni-binding peptide thereof do not seem to constitute part of the antigenic determinant for the T23 receptor. This is demonstrated in Fig. 2B because NiSO_4 and HSA-Ni were presented comparably well by live or glutaraldehyde-fixed APC, which lack Ag-processing capacity. The effectiveness of fixation was controlled using the murine T cell hybridoma D9/G5 (33), which secretes IL-2 in response to trinitrophenyl (TNP)-modified keyhole limpet hemocyanin. Fixation of syngeneic APC with glutaraldehyde under conditions used in Fig. 2 re-

duced TNP-modified keyhole limpet hemocyanin-specific IL-2 release by 92.5% (data not shown).

We also considered the possibility that HSA-Ni may carry Ni^{2+} ions to the APC surface where it might transfer them to metal coordination sites on MHC molecules. Again, this apparently too simplistic model is not supported by experimental data. As also shown in Fig. 2B, in contrast to APC that were pulsed and washed with NiSO_4 , APC pulsed with HSA-Ni were totally unable to stimulate T23 cells.

T cell activation by xenogenic metalloproteins

To assess the influence of the primary structure of HSA on the specificity of T cell responses to HSA-Ni, we tested the stimulatory capacity of Ni complexes with serum albumins derived from a variety of different species. Commercially available samples of DSA, BSA, CSA, PSA, or MSA were loaded with NiSO_4 and dialyzed as described for HSA-Ni. All preparations, including HSA-Ni, were used to stimulate five representative HSA-Ni-induced T cell clones of donor IF, using autologous EBV-transformed B cells as APC. As shown in Table II, the strongest stimulation of IF clones was obtained with HSA-Ni in all cases. However, BSA-Ni, CSA-Ni, PSA-Ni, and MSA-Ni also induced notable proliferation of all clones. In contrast, negligible stimulation was observed with DSA-Ni. Experiments performed with T23 cells and 18 μM albumin concentrations in serum-free medium, resulted in IL-2-specific CTLL proliferation of $19,544 \pm 3,788$ cpm for HSA-Ni, $6,285 \pm 467$ cpm for BSA-Ni, and $5,702 \pm 788$ cpm for PSA-Ni. Again, the effect of DSA-Ni (129 ± 58 cpm) was indistinguishable from Ag-free controls (133 ± 38 cpm).

These findings qualitatively, although not in all cases quantitatively, correlate with the Ni content of the different albumin preparations. As also demonstrated in Table II, atomic absorption spectroscopy revealed molar ratios of Ni to protein of 1.2 for HSA-Ni, and of 0.2 and 0.3 for CSA-Ni and PSA-Ni, respectively, but <0.01 for DSA-Ni. The lack of Ni binding to DSA has also been reported by others and appears to relate to the missing histidine in position 3 of the N-terminal sequence (34, 35) as compared with HSA or BSA (Table II). However, we found Ni to bind, although less efficiently, also to CSA and PSA (Table II), despite the fact

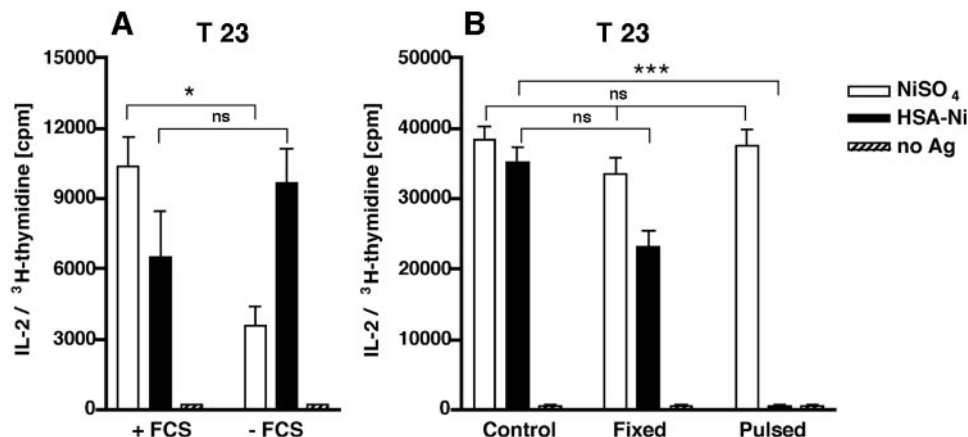


FIGURE 2. Activation of hybridoma T23 is influenced by serum factors and shows processing independent reactivity to HSA-Ni on fixed APC. A, T23 cells (5×10^4 cells/well) were activated with HSA-Ni (1 mg/ml) or NiSO_4 (2.5×10^{-4} M) for 24 h in the presence of irradiated B cells (5×10^4 cells/well), with or without 10% FCS-containing medium, and supernatants were used for IL-2 measurement in an IL-2-dependent CTLL proliferation bioassay. B, X-irradiated APC (6000 rad) were either untreated (control) or fixed with glutaraldehyde (fixed) and used to stimulate T23 cells in the presence of HSA-Ni (1 mg/ml) or NiSO_4 (10^{-4} M). In a third experiment, APC were pulsed for 1 h with HSA-Ni or NiSO_4 (see *Materials and Methods*), washed, and then used for stimulation (pulsed). For IL-2 assay and statistical analysis, see *Materials and Methods*.

that His₃ is missing also in these proteins. Therefore, it is intriguing to speculate whether Ni binding to CSA and PSA might be rather due to an as-yet-undefined second binding site that has recently been proposed for HSA and BSA (13, 36, 37).

To gain more insight into the relative affinities of Ni for different serum albumins, we determined their binding to Ni-covered chips by SPR (Biacore). The results of the Biacore analysis (Fig. 3A) also correlate well with the albumins' biological activities (Table II) in that the strongest binding was observed for HSA, reduced but significant binding was observed for BSA, CSA, PSA, and MSA, and almost no interaction was observed for DSA. The difference between HSA and DSA is most impressive when the kinetics of binding are compared (Fig. 3B).

The interaction of albumin with Ni²⁺ complexed via several of their coordination sites to linker molecules on a planar surface in the Biacore system in a way resembles the recognition of Ni by TCR on the MHC surfaces. This type of interaction obviously differs from the binding of proteins to free metal ions, which offer all possible coordinations for complexation. Consequently, the Biacore-determined binding constant of 7.5×10^{-4} M (Fig. 3C) is lower than most previously reported affinities for albumin-Ni interactions (38). It seems reasonable to assume that the contribution of Ni to the binding affinity between TCR and MHC might be in this order of magnitude.

Transfer of Ni²⁺ from HSA to histidine-containing peptides

Based on the failure to pulse APC with HSA-Ni (Fig. 2B), we concluded that Ni is not arbitrarily transferred from HSA to cellular surface proteins. In contrast, it has been reported that Ni may be transferred from HSA to histidine in solution (39). We also found that cocubation of HSA-Ni with either a dihistidyl peptide or the N-terminal tetrapeptide of HSA and subsequent dialysis, already at 1:1 molar ratios, resulted in the significant loss of HSA-

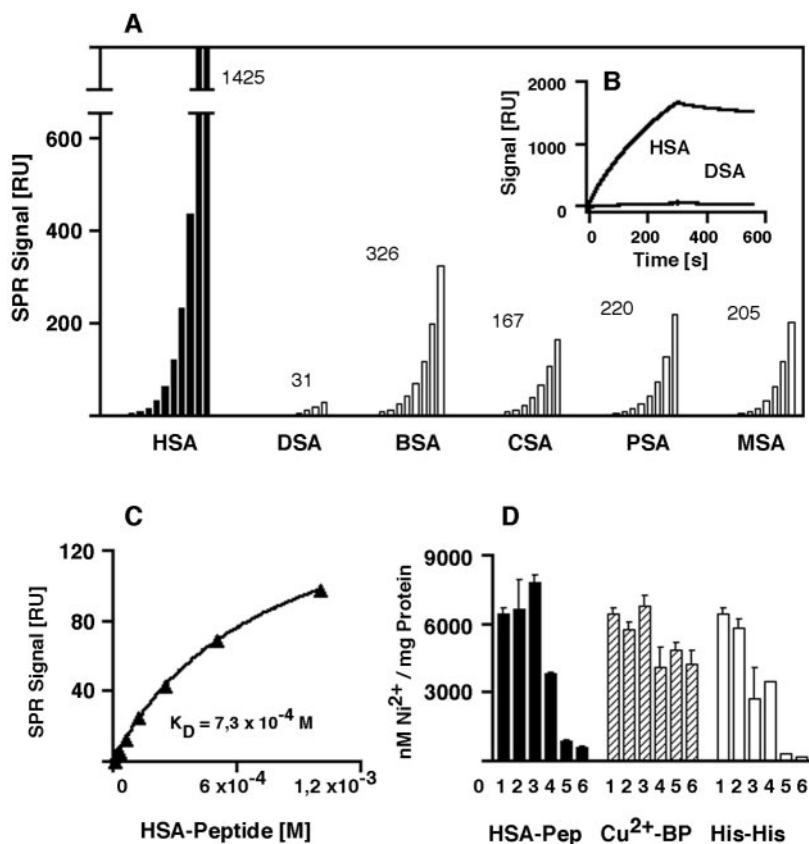
associated Ni (Fig. 3D). In contrast, the copper binding peptide Gly-Gly-His (40, 41) barely affected the Ni content of HSA-Ni under identical conditions at pH 7.4 (Fig. 3D). Hence, it appears likely that Ni will be transferred from HSA to other ligands, e.g., on cellular surfaces, only if binding sites of similar or higher affinity than HSA are offered. MHC molecules, and HLA-DR52c in particular, do not seem to fall into this category, as suggested by the results obtained using pulsed APC (Fig. 2B).

HSA is efficiently internalized by B cells and DC at 37°C

To determine whether HSA was attached at all to APC upon pulsing (Fig. 2B), we studied the interaction of fluorescein-labeled HSA (HSA-FITC) with the EBV-transformed B cell line WT47 by flow cytometry (FACS). The data in Fig. 4A reveal for the B cell line WT47 an increasing fluorescence signal with temperature, indicating energy-dependent uptake rather than binding of HSA-FITC. This uptake is concentration dependent (Fig. 4B) and reaches plateau levels after 20–30 min (Fig. 4C).

Comparable uptake of HSA-FITC was also observed for the vast majority of cells in peripheral blood, including CD19⁺ peripheral B cells (data not depicted), and for in vitro-generated human DC (Fig. 4, D–G) differentiated from peripheral blood monocytes (26, 27). In all cases, the uptake of HSA-FITC was strongly reduced at low temperature (Fig. 4, D and F), whereas at 37°C, iDC (E) accumulated dramatically more fluorescence than the mature population (G), again stressing endocytotic processes. For final proof, we analyzed the cell lines WT47 and Raji as well as human iDC, treated with HSA-FITC or HSA-Ni-FITC, by confocal scanning microscopy (Fig. 5). The low amount of HSA-FITC bound to cells at 4°C (Fig. 4) was attached to the cell surface (data not depicted). In contrast, at 37°C, practically all HSA-FITC was located inside the cells (Fig. 5, B, C, E, and F), with only trace amounts still colocalized with the B cell surface marker CD19 (C and F). No

FIGURE 3. SPR analysis of the interaction of different albumins to the Ni-NTA sensorchip (Biacore) and specific protein-peptide shuttling of HSA-bound Ni²⁺. *A*, Species-specific albumins were stepwise (1:2) diluted from 4×10^{-5} to 2×10^{-8} M in running buffer (10 mM HEPES, 150 mM NaCl, 50 μ M EDTA, 0.005% p20 (pH 7.4)), and the interaction of each albumin molecule (mobile phase) to Ni²⁺ immobilized on a Ni-NTA sensorchip (Biacore) was measured. Analysis was performed at a flow rate of 30 μ l/min. *B*, For the most divergent Ni-binding molecules, HSA and DSA, time-dependent association and dissociation curves to Ni-NTA sensorchip are shown, both at 4×10^{-5} M. *C*, Steady-state analysis of N-terminal HSA peptide interaction with the Ni-NTA sensorchip resulted in a K_D of 7.3×10^{-4} M. *D*, Metalloprotein HSA-Ni (120 μ M) was incubated for 6 h at 37°C, with ascending concentrations (0, 3, 20, 120, 500, and 720 μ M; labeled 1–6, respectively) of the N-terminal HSA peptide Asp-Ala-His-Lys (HSA-Pep), the known Cu-binding peptide Gly-Gly-His (Cu²⁺-BP), or the dipeptide His-His. After extensive dialysis, molar Ni concentrations bound to HSA were determined by atomic absorption spectrometry as described in *Materials and Methods*.



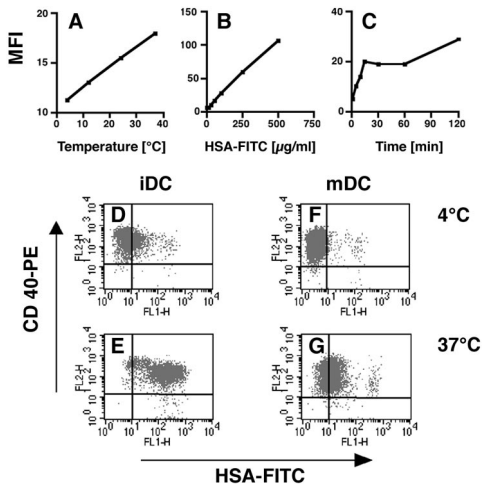


FIGURE 4. Uptake of HSA-FITC by human B cells and DC. Mean fluorescence intensity (MFI) of the B cell line WT47 was recorded in dependence of temperature (A), concentration (B), and time (C). A, Incubation with 500 $\mu\text{g/ml}$ HSA-FITC for 1 h at different temperatures (4, 12, 24, and 37°C). B, Incubation for 2 h at 37°C with different concentrations of HSA-FITC (10, 50, 100, 250, and 500 $\mu\text{g/ml}$). C, Incubation with 500 $\mu\text{g/ml}$ HSA-FITC and for different time intervals. D–G, iDC (D and E) and mDC (F and G) were incubated at 4 or 37°C with 500 $\mu\text{g/ml}$ HSA-FITC for 30 min. The subpopulations with different in HSA uptake in D–G may indicate a not completely synchronous *in vitro* maturation of DCs.

difference in this distribution was observed when the Ni-saturated preparation HSA-Ni-FITC was used (Fig. 5, H, I, K, and L). The fact that accumulation of HSA-Ni within APC upon pulsing at 37°C did not result in functionally detectable Ni determinants on the cell surface (Fig. 2B) stresses the notion that intracellular processing of HSA-Ni is neither required nor sufficient for Ni presentation to T cells.

Determination of surface-bound Ni^{2+} on B cells with NPG

The lack of functional Ni determinants on APC pulsed with HSA-Ni (Fig. 2B) and confocal microscopy data in Fig. 5 indicated that the transport of Ni via HSA-Ni may be directed to the interior rather than to the surface of APC. However, these data did not answer the question whether Ni^{2+} might be retained on the cell surface by proteins other than MHC. To answer this question, we made use of the fluorescent dye NPG, which has been used for quantitative determinations of Ni^{2+} and Zn^{2+} in aqueous solutions (42, 43).

Because the NPG preparation used is known not to permeate cellular membranes (30), we attempted to use it in FACS staining to detect surface-bound Ni^{2+} . We show in Fig. 6 that addition of NPG to human Raji cells, indeed, results in significantly enhanced cellular fluorescence when NiSO_4 or HSA-Ni are present in the medium at 10 or 500 μM concentrations (Fig. 6, C–F). Controls in the presence of Ni-free HSA (Fig. 6A) or 10 μM CuSO_4 (B) show the same background fluorescence as NPG alone (dotted lines). Removal of surplus Ni by washing of the cells reduced the fluorescence to background values in all cases where Ni was added at 10 μM concentration (Fig. 6, I and K). However, cells washed after incubation with 500 μM NiSO_4 retained much of their fluorescence (Fig. 6J), indicating surface-bound Ni^{2+} . In contrast, treatment with 500 μM HSA-Ni barely left any detectable Ni on the cellular surface after washing (Fig. 6L). These data may explain why pulsing with NiSO_4 but not with HSA-Ni creates stimulatory Ni/MHC determinants on APC. It thus appears that the internalization of HSA-Ni removes Ni^{2+} from the interaction with

specific T cells, again stressing the point that processed peptides of HSA are not part of the antigenic determinants for clone ANi 2.3 or hybridoma T23. In contrast, constitutive presence of HSA-Ni in the medium (Fig. 6, E and F), probably representing physiological conditions of Ni presentation, may very effectively transfer Ni^{2+} to high-affinity coordination sites within TCR-MHC contacts.

Discussion

Human hypersensitivity to Ni, like contact allergies in animal models, is mediated by allergen-specific T cells (1, 2, 20). HLA-restricted Ni-reactive T cell lines and clones of both CD4 and CD8 phenotypes have repeatedly been isolated by NiSO_4 or NiCl_2 stimulation of T cells from peripheral blood or skin lesions of allergic patients and even from nonsensitized individuals (1, 9, 44, 45). Recent data imply that Ni^{2+} mediate TCR/MHC contact by formation of complexes involving amino acids of TCR and/or MHC as coordination partners (24, 46, 47). Ni-peptide complexes have been extensively studied, and histidine has been identified as a preferred, but by no means the only coordination partner for Ni^{2+} (15, 38, 48, 49). In the outer layers of human skin, notably in the stratum corneum, histidine-rich proteins such as filaggrin (11, 12, 50) might, therefore, be expected to effectively capture free metal ions at their point of entry and prevent or mediate their further migration. In this scenario, a loading of LC with Ni would require protein carriers that not only bind Ni, but also shuttle it to deeper layers of the epidermis. One candidate carrier molecule is HSA, which contains a binding site for Cu and Ni, defined by its four N-terminal amino acids Asp-Ala-His-Lys (14). Approximately 40% of the body's extravascular HSA content is located within the skin, which is 2-fold more concentrated per weight than in muscle. Both, the presence of HSA in sweat (14, 51) and the changes in human skin during hydration, indicate its bidirectional trafficking through the epidermal basement membrane (19).

We have loaded HSA with Ni by incubation with NiSO_4 and subsequent dialysis and have determined a molar ratio of Ni:HSA of $\sim 1:1$ by atomic absorption spectroscopy. The HSA-Ni complex, which may also play an important role in patients suffering from occupational asthma (52, 53), could be used similarly to NiSO_4 to stimulate Ni-reactive CD4^+ T cells from blood mononuclear cells of nickel allergic patients. All T cell clones derived from HSA-Ni stimulation reacted to stimulation with NiSO_4 (Table I). Most importantly, equimolar Ni concentrations added in the form of either NiSO_4 , NiCl_2 , or HSA-Ni all induced indistinguishable proliferative stimuli for the NiSO_4 -induced human T cell clone SE9 (Fig. 1). In contrast, several of the Ni-reactive T cells that were induced by NiSO_4 reacted only poorly to HSA-Ni (Table I), possibly reflecting the lower (15 μM) Ni concentration in HSA-Ni vs NiSO_4 (100 μM) stimulation. The present study concentrates on the majority of clones that react to HSA-Ni as well as to NiSO_4 . Most of the experiments were performed using the intensively characterized Ni-reactive murine T cell hybridoma T23, which expresses the human TCR of clone ANi 2.3 (24, 28). For hybridoma T23, we show (Fig. 2A) that removal of serum from the medium reduces the effectiveness of NiSO_4 to activate its TCR, indicating that Ni^{2+} may actually require intermediate complexation to HSA or other serum constituents for optimal stimulation. In contrast, the stimulatory capacity of HSA-Ni is reduced by the addition of serum, presumably due to dilution of HSA-Ni with Ni-free albumin.

In analogy to T cell responses against other haptens (54), one might expect that HSA-Ni is taken up and processed by APC, and that Ni complexed to the N-terminal HSA peptide is presented to T cells on MHC molecules. However, for several reasons, the scenario is more analogous to the situation of noncovalent TCR-MHC

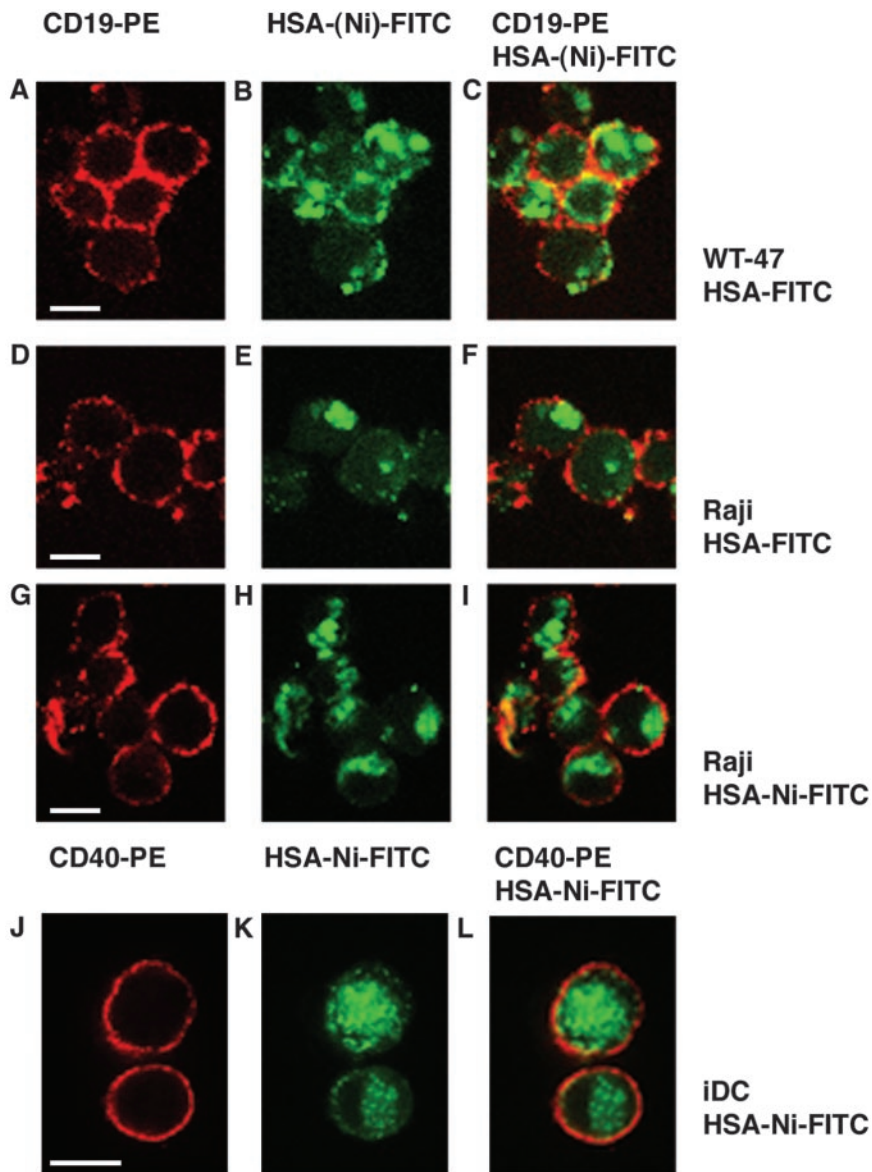


FIGURE 5. Cytoplasmic localization of HSA-FITC and HSA-Ni-FITC in human B cells and human iDC after incubation at 37°C. *A–I*, B cells were incubated with Ni-free albumin (HSA-FITC; 500 $\mu\text{g}/\text{ml}$) or Ni-complexed albumin (HSA-Ni-FITC; 500 $\mu\text{g}/\text{ml}$) for 1 h at 37°C. To control intracellular uptake, B cells were stained with B cell marker anti-CD19-PE at 4°C, as described in the FACS protocol. After washing, cells were fixed and visualized by confocal laser scanning microscopy. *C*, *F*, and *I*, The overlay of both stains demonstrates cytoplasmic but almost no extracellular membrane-bound localization of HSA-FITC and HSA-Ni-FITC and a cell surface signal for the B cell marker CD19. *J–L*, iDC were incubated with Ni-complexed albumin (HSA-Ni-FITC; 500 $\mu\text{g}/\text{ml}$) for 1 h at 37°C. To control intracellular uptake, iDC were stained with anti-CD40-PE, as described above. After washing, cells were fixed and visualized by confocal laser scanning microscopy. *L*, The overlay of both stains demonstrates cytoplasmic but almost no extracellular membrane-bound localization of HSA-Ni-FITC in human iDC. The intracellular localization of HSA-FITC has not been studied further.

contacts by chemically inert drugs such as sulfamethoxazole (55, 56). First, we show that T cells reactive to HSA-Ni react with complexes of Ni with albumins from various different species. Their stimulatory capacities correlate with their relative binding affinities for Ni (Fig. 3) as well as with the molar ratio of Ni remaining associated to the proteins upon extensive dialysis (Table II). Second, HSA-Ni as well as the other Ni-saturated albumins stimulate T cells from genetically unrelated individuals (Table I, and C. Moulon and H. J. Thierse, unpublished data). Third, blocking of intracellular protein processing by glutaraldehyde fixation of APC does not inhibit Ni presentation to T cells via HSA-Ni (Fig. 2B). Furthermore, HSA-Ni is effectively internalized by APC at 37°C, with only small amounts of HSA remaining attached to the cell surface upon washing. Such pulsed APC do not contain measurable amounts of Ni on their surface (Fig. 6L) and, most importantly, do not stimulate the same Ni-reactive T cells that they activate when HSA-Ni is present in the medium (Fig. 2B). Hence, the MHC-Ni determinants recognized by the TCR structures under study are neither produced by intracellular processing of HSA-Ni, nor does HSA-Ni transfer recognizable numbers of Ni²⁺ ions to hypothetical binding sites on MHC. This stands in contrast to successful pulsing of APC with carrier-free NiSO₄ (Fig. 2B), reveal-

ing that Ni binding sites do, in fact, exist on MHC molecules. However, the MHC probably exhibits only some of the necessary Ni-coordination sites and, therefore, lower affinity for Ni²⁺ than the complete binding site in HSA. Thus, transfer of Ni from HSA to MHC/peptide appears rather unlikely. This view is corroborated by data in Fig. 3D, revealing that transfer of Ni²⁺ from HSA to other peptides requires optimal Ni binding motifs in these molecules.

The current concept of Ni recognition by TCR (24, 46, 47) envisages Ni²⁺ as bridging MHC and TCR, with both structures contributing coordination sites. This view implies that a geometric arrangement of at least four sterically optimized coordination sites for Ni²⁺ may only be provided by the combination of TCR and MHC. Due to positive selection during thymic development, low affinity and, therefore, short-lived nonproductive contacts between TCR and MHC are expected to occur even in the absence of Ag. The permanent presence of free or HSA-bound Ni²⁺ ions in proximity to such contact zones would allow their immediate insertion into these sites, stabilizing the TCR/MHC contact to an extent that it facilitates T cell activation. In that situation, the high-affinity complex of HSA-Ni has the advantage of releasing Ni²⁺ selectively into these short-lived TCR/MHC-defined coordination sites,

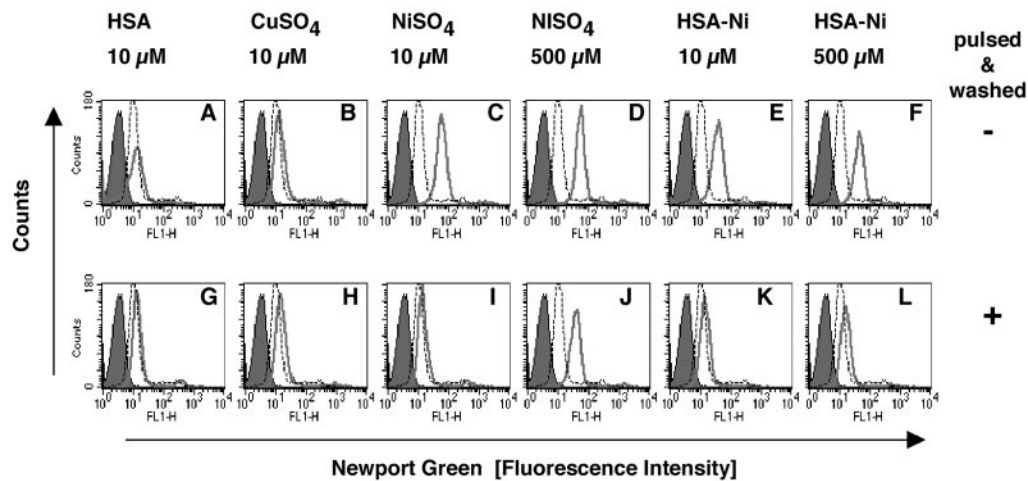


FIGURE 6. NPG specifically recognizes Ni^{2+} on B cell membrane surfaces. A–L, Human B cells (Raji) were washed with 0.9% (w/v) NaCl and incubated with Ni, Cu, albumin, and the metalloprotein HSA-Ni at the concentrations as indicated, for 1 h at 37°C. Following extensive washing (G–L) or no washing (A–F), cells were incubated for 30 min with NPG (1 μM) and analyzed by FACS. Dotted lines, NPG alone; continuous lines, NPG in the presence of the tested reagent; black filled histograms, no NPG.

avoiding attachment of Ni^{2+} to lower affinity, eventually even competitive binding sites.

One may also consider that CD43, which has been proposed as a potential cellular binding site for HSA (57), may be involved on T cells in bringing HSA-Ni into proximity with TCR or TCR/MHC contact sites. In addition, conformational changes of the HSA molecule induced by binding to receptors or by metabolically induced modifications may lower the N-terminal affinity for Ni^{2+} , facilitating an exchange of the metal ions to other high-affinity coordination sites. The flexibility of the N-terminal HSA domain is stressed by crystallography studies (58). In fact, the N-terminal binding affinity of HSA for Cu^{2+} has been shown to be affected by oxidation of the remote cysteine residue 34 (59, 60). Interestingly, oxidative processes are hallmarks of inflamed skin.

In conclusion, the composition of human skin makes it very likely that Ni^{2+} ions set free upon contact with Ni-containing alloys will immediately be complexed and locked within the outermost regions of the skin. Ni presentation to T cells by LC, therefore, requires carrier molecules that are capable of eluting Ni^{2+} from such stores and of shuttling back and forth through the epidermal basement membrane. The evidence presented in this report reveals that HSA is a prime candidate for such a transfer system and may even add specificity in terms of directing Ni^{2+} to adequate TCR/MHC conjugates.

Acknowledgments

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