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Nickel Allergy in Mice: Enhanced Sensitization Capacity of Nickel at Higher Oxidation States

Suzan Artik,2,3,*,† Christian von Vultee,3,* Ernst Gleichmann,* Thomas Schwarz,‡ and Peter Griem*

Attempts to induce contact hypersensitivity to nickel in mice using, e.g., Ni(II)Cl₂ often failed. Here, we report that sensitization was achieved by injecting Ni(II)Cl₂ in combination with either CFA or an irritant, such as SDS and PMA, or IL-12, or by administering nickel at higher oxidation states, i.e., Ni(III) and Ni(IV). Although Ni(II), given alone, was ineffective in T cell priming, it sufficed for eliciting recall responses in vivo and in vitro, suggesting that Ni(II) is able to provide an effective signal 1 for T cell activation, but is unable to provide an adequate signal 2 for priming. Immunization of mice with nickel-binding proteins pretreated with Ni(IV), but not with Ni(II), allowed them to generate nickel-specific CD4⁺ T cell hybridomas. Ni(II) sufficed for restimulation of T cell hybridomas; in this and other aspects as well, the hybridomas resembled the nickel-specific human T cell clones reported in the literature. Interestingly, restimulation of hybridomas did not require the original Ni(IV)-protein complex used for priming, suggesting either that the nickel ions underwent ligand exchange toward unknown self proteins or peptides or that nickel recognition by the TCR is carrier-independent. In conclusion, we found that Ni(III) and Ni(IV), but not Ni(II) alone, were able to sensitize naive T cells. Since both Ni(III) and Ni(IV) can be generated from Ni(II) by reactive oxygen species, released during inflammation, our findings might explain why in humans nickel contact dermatitis develops much more readily in irritated than in normal skin. The Journal of Immunology, 1999, 163: 1143–1152.

Nickel is a metal that is used in many different alloys. It occurs, for example, in coins, buttons, costume jewelry, and items made out of stainless steel or aluminum. Its widespread use contributes to the fact that nickel is one of the most common contact allergens (1). Nickel-specific T cells have been detected in sensitized humans and animals. These T cells could be restimulated by Ni(II) in vitro (2–4) and, upon adoptive transfer, in vivo (5). Paradoxically, however, the mechanism underlying de novo sensitization to this common contact allergen is obscure. In the two studies reporting on successful sensitization of mice to nickel, special measures had to be taken, such as breeding and rearing the animals in a nickel-free environment (5) or administering exceedingly high concentrations of nickel salts (6). In contrast, most previous attempts to experimentally sensitize mice to nickel failed (7–11).

In trying to induce sensitization to nickel in mice, we took into consideration the clinical experience that, in humans, allergic contact hypersensitivity to nickel develops much more readily in inflamed rather than normal skin. Here, we report that, when skin inflammation is mimicked in the mouse by coadministration of Ni(II)Cl₂ with either an irritant, CFA, or IL-12, contact hypersensitivity to Ni(II), indeed, ensues. In inflammation, reactive oxygen species, such as hydrogen peroxide (H₂O₂) (4) and hypochlorite (OCl⁻)₄, are produced by phagocytes. These powerful oxidants can oxidize Ni(II), i.e., nickel in the oxidation state +2, to the higher oxidation states Ni(III) and Ni(IV), respectively (12, 13). The higher nickel oxidation states possess a far greater chemical reactivity than Ni(II). Up until now, however, no study has investigated the possible significance of higher oxidation states in allergic contact hypersensitivity to nickel.

When assessing the sensitization potential of different gold compounds, our group has shown that gold(I) has a poor sensitizing capacity whereas the higher oxidation state, gold(III), due to its superior chemical reactivity, proved to be a potent sensitizer (14–17). In this context, it is worth noticing that OCl⁻ can oxidize gold(I) to gold(III) (15, 18). By analogy to gold, here we asked whether the higher oxidation states of nickel would be more potent than Ni(II) in inducing de novo sensitization to this heavy metal. Two independent experimental approaches were made to assess the sensitizing capacity of higher nickel oxidation states. In the first approach, Ni(III) and Ni(IV), respectively, were used for priming mice, and specific T cell responses were assessed in vivo using the mouse ear swelling test (MEST) and the popliteal lymph node (PLN) assay. In the second approach, Ni(IV) complexed with protein was used for priming, and nickel-specific CD4⁺ T cell hybridoma clones were established and characterized.

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4 Abbreviations used in this paper: OCl⁻, hypochlorous acid; HSA, human serum albumin; MEST, mouse ear-swelling test; MSA, mouse serum albumin; Ni(IV)(OH)₂O, nickel oxide hydroxide; PLN, popliteal lymph node; DC, dendritic cell.
Materials and Methods

Mice
Specific pathogen-free female C57BL/6J (H-2^d) mice obtained from Harlan Olac (Bicester, U.K.) were used throughout. Animals were used at 9–20 wk of age at the onset of the experiments. They had free access to drinking water and standard rodent lab chow (No. 1324, Altromin, Lage/Lippe, Germany). No measures were taken to protect the animals from exposure to nickel: cages (made from plastic) were covered by stainless steel covers, and drinking water was provided by glass bottles covered with water outlets made from stainless steel.

Cell lines
Thymoma line BW5147 (TCR ß^+^) (19) was kindly provided by Dr. H.-G. Burgert (Max Planck Institute for Immunobiology, Freiburg, Germany).

Reagents
AuI(TM) (dissodium aurothiomalate) was kindly provided by E. Trosse (Hamburg, Germany). In addition, the following metal salts were used: ZnCl_2, CoCl_2·6H_2O, and K_2Cr_2O_7 were purchased from Sigma-Aldrich Chemie (Deisenhofen, Germany); Na_2PtCl_4, Na_2PdCl_4, Na_2PdCl_6, and Pt(NH_3)_4Cl_2 were gifts from Degussa (Frankfurt/Main, Germany); H_2AuCl_4, CuSO_4·5H_2O, FeCl_3·6H_2O, and NiCl_2·6H_2O and Ni(II)Cl·6H_2O were purchased from E. Merck (Darmstadt, Germany). Sterile, pyrogen-free 0.9% saline was purchased from Fresenius (Bad Homburg, Germany). Nitroso-benzene was purchased from Sigma-Aldrich; before use, it was dissolved in absolute ethanol (Merck) and diluted in 0.9% saline to the concentration indicated. H_2O_2 (30%) was purchased from Merck. SDS was obtained from Sigma-Aldrich. Recombinant murine IL-12 was kindly provided by S. Wolf (Genetics Institute, Cambridge, MA). For in vivo injection, IL-12 was diluted in sterile endotoxin-free saline and injected i.p. at a dose of 500 µg/kg.

Ni(III) and Ni(IV) were freshly prepared for each experiment because they are rather unstable and not available commercially. Ni(III) was prepared by mixing Ni(II)Cl_2 with a 30-fold molar excess of H_2O_2. Ni(IV) was prepared by oxidation of Ni(II) through OCl^- ions. In both cases, the solutions were neutralized with diluted NaOH.

MEST
A modification of the MEST was used, as described by van Hoogstraten et al. (5). For induction of sensitization, groups of mice were injected intradermally with 50 µl into both flanks, each injection containing either 1 µmol Ni(II)Cl_2 in 0.9% saline, 2) a mixture of 1 µmol Ni(II)Cl_2 and 30 µmol H_2O_2, i.e., Ni(III), in saline, 3) 30 µmol H_2O_2 in saline, or 4) 1 µmol Ni(III)Cl_2 in saline mixed 1:1 with CFA. In the case of irritants, the intradermal injections, 50 µl each, into both flanks were administered subsequently, as follows: 2) SDS in saline given 4 h before 1 µmol Ni(II)Cl_2, or 600 ng PMA given 1 h before 1 µmol Ni(II)Cl_2. In the case of IL-12, i.p. injection of 500 µl of saline or 500 µl of a mixture of 7.35 µmol Ni(II)Cl_2 and 300 mM final concentration, respectively, or of either substance alone, were injected s.c. into the left hindfoot pad on day 0. For determination of primary responses, mice (five to six animals per group) were killed on day 6, and both PLNs were removed. Cell numbers of each PLN were determined, and the PLN cell count index was calculated by dividing the value obtained from the draining (ipsilateral) PLN through that of the control (contralateral) PLN.

Murine popliteal lymph node assay
The popliteal lymph node (PLN) assay was performed as described (24). Briefly, 50 µl of saline containing H_2O_2 and Ni(II)Cl_2 at the concentrations indicated, or of either substance alone, were injected s.c. into the left hindfoot pad on day 0. For determination of primary responses, mice (five to six animals per group) were killed on day 6, and both PLNs were removed. Cell numbers of each PLN were determined, and the PLN cell count index of each animal was calculated by dividing the value obtained from the draining (ipsilateral) PLN through that of the control (contralateral) PLN. For induction of secondary responses, on day ~ 10, mice were primed by s.c. injection into the left hindfoot pad 50 µl of either a mixture of 7.35 µmol H_2O_2 and 0.25 µmol Ni(II)Cl_2 in saline, each substance alone, or saline alone. On day 0, mice were challenged for recall by s.c. injection into the right hindfoot pad of either 0.25 µmol Ni(II)Cl_2 in 50 µl saline, or saline alone. On day 6, mice were killed, and their PLN cell count indices were determined. In control experiments with nitroso-benzene, the doses used for priming and challenge were 50 nmol and 3 nmol nitroso-benzene, respectively. The latter dose was termed “suboptimal” because it was too small for priming, but sufficient for eliciting a secondary PLN response.

Ags used for generation of T cell hybridomas
Mouse serum albumin (MSA), human serum albumin (HSA), and bovine ribonuclease A (RNase A) were purchased from Sigma. For complex formation with Ni(II), MSA and HSA were incubated for 1 h with a 100-fold molar excess (2.9 mM) of Ni(II)Cl_2 in 0.9% saline; the final protein concentration was 2 mg/ml. Complex formation of the proteins with Ni(IV)(OH)O was conducted as follows: to a solution of the respective protein in 0.9% saline (concentration 2 mg/ml), a 100-fold molar excess (2.9 mM in the case of MSA and HSA) or 55-fold molar excess (7.9 mM in the case of RNase) of Ni(IV)(OH)O was added. Diluted HCl was added up to complete dissolution of the Ni(IV) compound. After 1 h of incubation, the solution was neutralized with diluted NaOH.

T cell hybridomas
Mice were immunized by s.c. injection at the base of tail with 100 µg of the indicated Ag emulsified in CFA (1:1). After 8–10 days, spleens were removed, and single-cell suspensions were prepared in PBS. After lysis of erythrocytes in hypotonic buffer (17 mM Tris-HCl (pH 7.2) and 160 mM NH_4Cl) and enrichment of splenic T cells by nylon wool separation, T cells (2 × 10^6/ml) as APC. After 3 days, living cells were isolated by gradient centrifugation on Ficoll-Paque (Pharmacia, Freiburg, Germany) and expanded in culture medium for 3 days in the presence of 20 U/ml rIL-2. T cell blasts isolated by Ficoll-gradient centrifugation were frozen with BW5174 tumor cells using polyethylene glycol 1500 (Boehringer, Mannheim, Germany), as described by the manufacturer. After selection for hybrid cells in hypoxanthine/aminopterin/thymidine (HAT) medium for 2 wk, cells were cultured in medium containing hypoxanthine and thymidine. T cell hybridomas reacting to Ag in the stimulation assay described below were subcloned by limiting dilution.

Ag-induced stimulation of T cell hybridomas was tested in standard IL-2 release assays. For this purpose, hybridomas (1 × 10^3/200 µl supplemented RPMI medium) were stimulated with either native protein (MSA, HSA, or RNase), Ni(IV)(OH)O-pretreated protein, Ni(III)-pretreated protein, or Ni(II)Cl_2 alone. For screening of hybridomas, a protein concentration of 0.1 mg/ml and a nickel concentration of 100 µM were used. All assays were performed in the presence of syngeneic spleen cells

Porliferation of auricular lymph node cell
In addition to determining ear swelling after recall with Ni(II)Cl_2, proliferation in vitro of auricular lymph node cells was measured. Lymph node cells were obtained 48 h after challenge, and 3 × 10^5 cells were incubated for 16 h in the presence or absence of 100 µM Ni(II)Cl_2 in RPMI 1640 medium (Life Technologies, Paisley, U.K.) supplemented with gentamicin, penicillin, streptomycin, essential and nonessential amino acids, 10% FCS, and 50 µM 2-ME. Cell proliferation was determined by adding 18.5 kBq [3H]thymidine at the beginning of the culture, harvesting the cells onto nitrocellulose filter, and measuring incorporated radioactivity in a scintillation counter.

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either no Ni(II)Cl₂ or Ni(II)Cl₂ (100 μmol) were added to each of two groups of mice and injected intradermally into each flank with (A) 1 μmol Ni(II)Cl₂ in saline after injection of 2% SDS 4 h earlier or with either of these substances alone. 1 μmol Ni(II)Cl₂ in saline, or saline alone, after pretreatment of mice with 2 × 10⁵ g/ml of anti-MHC class II mAb (clone M5/114; PharMingen, Hamburg, Germany) for control.

Analysis of TCR by flow cytometry

The following anti-mouse FITC-conjugated mAb, all purchased from PharMingen, were used to determine the TCR Vα and Vβ repertoire of nickel-specific T cell hybridoma clones: panαβ (HS7-597), Vα2 (B20.1), Vα3.2b (R3-16), Vα8 (B21.14), Vα11b.d (R88-1), Vβ2 (B20.6), Vβ4 (KT4), Vβ5.1.5 (MR9-4), Vβ6 (RR4-7), Vβ7 (TR310), Vβ8.1.8.2 (MR5-2), Vβ8.3 (IB3.3), Vβ9 (MR10-2), Vβ10b (B21.5), Vβ11 (R3-15), Vβ12 (MR11-1), Vβ13 (MR12-3), Vβ14 (14-2), and Vβ17a (KJ23); mAb Vβ3 (KJ25) was PE conjugated. T cell hybridoma cells (10⁵ cells/20 μl PBS) were stained for 20 min at 4°C in 96-well plates. Fluorescence was measured using a FACScan flow cytometer and the CellQuest analysis program (Becton Dickinson, Mountain View, CA).

Statistical analysis

Statistical significance of results was determined by ANOVA tests. The level of significance was set at p ≤ 0.05. All experiments were performed at least twice to assure reproducibility.

Results

Secondary responses to nickel in the MEST

To mimic nickel contact with inflamed skin, mice were injected into the flanks with a 2% solution of the irritant SDS, while control mice received saline only. Two hours later, 1 μmol of Ni(II)Cl₂ was injected into the same skin areas on both flanks. Upon challenge with 0.2 μmol Ni(II)Cl₂ into the ears, only mice primed with Ni(II)Cl₂ plus SDS, but not Ni(II)Cl₂ or SDS alone, showed a significant increase in ear thickness (Fig. 1A). When other inflammation-inducing substances were employed, we observed that administration of PMA or CFA also was able to induce sensitization to Ni(II) (Fig. 1A).

IL-12 has been identified as a critical mediator in the induction of contact hypersensitivity (26, 27). To study whether IL-12 enables sensitization to Ni(II), mice received two i.p. injections of 500 ng rIL-12 before administration of Ni(II). As shown in Fig. 1B, pretreatment of mice with rIL-12 before injection of Ni(II) resulted in the priming of T cells for a nickel-specific secondary reaction.
Sensitization of mice by nickel at higher oxidation states

Since the application of irritants like SDS induces inflammatory infiltrates consisting of activated neutrophils and other phagocytes (28, 29) and, hence, release of reactive oxygen species, such as H₂O₂ and OCl⁻, we examined whether H₂O₂ itself can enhance sensitization to Ni(II)Cl₂. Mice were injected at the flanks with a mixture of Ni(II)Cl₂ and H₂O₂ that is known to result in the formation of Ni(IV) (12, 20). After priming with Ni(III), a secondary response upon challenge with Ni(II) was observed (Fig. 1C), whereas injection of Ni(II) or H₂O₂ alone did not result in a successful T cell priming (Fig. 2A). Significantly increased ear-swelling responses were obtained at 48 and 72 h after challenge, but not after 24 h (Fig. 2A). Similar results as with Ni(III) were found with Ni(IV); mice that had been primed with the higher nickel oxidation state Ni(IV) by injection of a suspension of Ni(IV)(OH)₂O showed a secondary response to Ni(II), as determined by MEST (Fig. 1C).

Specificity of the ear-swelling response was demonstrated by cross-cross immunization with K₂Cr₂O₇ (Fig. 2B), a common sensitiser that has also been studied in the MEST (5, 9). A significant ear-swelling response was seen in mice primed with K₂Cr₂O₇ and challenged with recall with K₂Cr₂O₇. By analogy, mice primed with Ni(III) responded to challenge with Ni(II). No cross-reaction between K₂Cr₂O₇ and Ni(II) was observed.

**Proliferation of auricular lymph node cells in vitro**

In some experiments, the proliferative response of cells from the draining lymph nodes also was determined. Auricular lymph node cells from mice that had been primed at the flanks with Ni(III) and challenged at the ears with Ni(II) showed enhanced spontaneous proliferation in vitro, indicating an ongoing secondary response. This effect became even more prominent when the lymph node cells were incubated in the presence of 100 μM Ni(II)Cl₂ (Fig. 2C). Thus, the proliferative response of cells from the draining lymph nodes corresponded with the results of the MEST performed in the donors of these lymph nodes.

**Primary and secondary responses to nickel in the PLN assay**

Unlike the MEST, which detects only secondary immune responses, the PLN assay, another well-established test for identification of sensitizing chemicals (24), allows detection of primary immune responses as well. Primary PLN responses were studied first. Mice were injected into one hind footpad with a mixture of H₂O₂ and Ni(II)Cl₂ to examine whether the combination would induce a stronger primary PLN response than each compound alone. As shown in Fig. 3A, 10 mM Ni(II)Cl₂ completely failed to induce PLN enlargement. When H₂O₂ was admixed to Ni(II)Cl₂, however, distinct PLN enlargement was observed. With all three H₂O₂ concentrations used, the PLN reaction to the Ni(II)Cl₂/H₂O₂ mixture, i.e., Ni(III) (12, 20), was significantly higher than the reaction caused by H₂O₂ alone.

Secondary PLN responses were studied by challenging mice by s.c. injection of Ni(II)Cl₂ into the right foot pad. A secondary response to nickel could be elicited only in mice that had been primed with Ni(III) (Fig. 3B). Notably, Ni(II) proved to be sufficient for eliciting specific secondary responses. Specificity of the response to Ni(II) was demonstrated in cross-cross immunization experiments using nitrosobenzene (Fig. 3C). Nitrosobenzene is a protein-reactive chemical known to elicit primary and specific secondary PLN responses. Only nitrosobenzene-primed mice exhibited a positive PLN response upon recall with nitrosobenzene, and only mice primed with Ni(III) responded to Ni(II) upon recall; there was no cross-reaction.

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5. M. Wulferink, J. Gonzalez, C. Goebel, and E. Gleichmann. T cells ignore antigen, a phosphatase, but respond to its reactive metabolites generated by phagocytes: possible implications for the pathogenesis of toxic oil syndrome (TOS). (Submitted for publication.)

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**FIGURE 2.** Coadministration of H₂O₂ enables induction of contact hypersensitivity to Ni(II). A. Groups of mice were injected intradermally into each flank with either 1 μmol Ni(II)Cl₂ plus 30 μmol H₂O₂, i.e., Ni(III), in saline, or with either of these substances alone. Ten days after priming, animals were challenged for recall by injection into each ear of either 0.2 μmol Ni(II)Cl₂ in saline. B. Groups of mice were primed by intradermal injection into each flank of either 1 μmol Ni(II)Cl₂ plus 30 μmol H₂O₂, i.e., Ni(III), either of these substances alone, or an emulsion of 1 μmol K₂Cr₂O₇ and CFA. Ten days after sensitization, animals were challenged for recall by injection into each ear of either 0.2 μmol Ni(II)Cl₂ or 70 nmol K₂Cr₂O₇ in saline. C. Recall response in vitro to Ni(II) by auricular lymph node cells obtained from groups of mice used in MEST. Lymph node cells from each group were pooled and cultured with 18.5 kBq [³H]thymidine for 16 h in the presence or absence Ni(II)Cl₂, as indicated. Data in A and B were obtained at 48 h after challenge; in C, mean cpm values of triplicates are shown. *, p < 0.05; **, p < 0.01; ***, p < 0.001. In A, asterisks denote differences in comparison with each other group; in B, asterisks denote differences between groups compared by brackets.
FIGURE 3. Injection of Ni(III) evokes a primary response and allows elicitation of Nickel-specific recall responses in the PLN assay. A, On day 0, groups of mice received a single s.c. injection into the left hindfoot pad of either saline (column at the very left), Ni(II)Cl₂ (i.e., Ni(III)), with either substance alone in saline, or saline alone. Ten days after immunization, mice were challenged for recall by s.c. injection into the right hindfoot pad of either 0.25 nmol Ni(II)Cl₂ plus the indicated concentration of H₂O₂ (black bars). On day 6, PLN cell counts were determined. B, Groups of mice were injected into the left hindfoot pad with a mixture of 7.35 μmol H₂O₂ and 0.25 μmol Ni(II)Cl₂ (i.e., Ni(III)), with either substance alone in saline, or with saline alone. Ten days after immunization, mice were challenged for recall by s.c. injection into the right hindfoot pad of either 0.25 μmol Ni(II)Cl₂ in saline, or saline only, as indicated. Six days later, mice were killed, and the PLN cell count index was determined. C, Groups of mice were primed by injection into the left hindfoot pad of either a mixture of Ni(II)Cl₂ plus H₂O₂ in saline (same doses as in B), either of these substances alone, or 50 nmol nitrosobenzene (NB). Two months later, when PLN indices of nitrosobenzene-treated mice had reverted to background level, mice were challenged for recall by injection into the right hindfoot pad of either 0.25 μmol Ni(II)Cl₂ or a suboptimal dose of nitrosobenzene (3 nmol). The PLN index was determined 6 days later. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001. In A and C, asterisks denote differences between groups compared by brackets; in B, in comparison with each other group.

The results described above (Figs. 1–3) raised two important questions. First, how certain can we be to have employed nickel at higher oxidation states? Second, how sure can we be that the successful priming observed with oxidized Ni(II) was indeed due to Ni(III) and Ni(IV), respectively, and not to an adjuvant effect of the oxidants used, i.e., H₂O₂ and OCl⁻? With regard to the first question, it should be realized that direct, physico-chemical detection of nickel at higher oxidation states is technically very demanding and has not been achieved up to now in a cell culture system, not to mention an animal; it was therefore not attempted in the present investigation. Instead, we took indirect approaches to ascertain that, indeed, higher oxidation states of nickel were used. On inspection, we noted disappearance of the characteristic green color of the Ni(II)-water complex after addition of the oxidants H₂O₂ and OCl⁻, respectively, and this was confirmed by UV-Vis spectroscopy. Addition of H₂O₂ to Ni(II)Cl₂, as used for the generation of Ni(III), resulted in partial disappearance, and addition of OCl⁻, as used for the generation of Ni(IV), resulted in complete disappearance of the green color. The green color, however, reappeared if thiol-containing reducing compounds, such as glutathione, were added (data not shown). Addition of OCl⁻ to Ni(II)Cl₂, followed by centrifugation, resulted in formation of a black precipitate that has been shown to contain Ni(IV), by means of infrared spectroscopy, x-ray photoelectron spectroscopy, and electron spin resonance (13). Hence, the combined evidence indicates that indeed we employed nickel at higher oxidation states.

With regard to the second question, the following experiments were undertaken to remove the oxidants added to Ni(II) for synthesis of the higher oxidation states of nickel and, thus, exclude a possible adjuvant effect of residual oxidant. In the case of H₂O₂, catalase was added to the mixture of Ni(II) and H₂O₂ to degrade excess H₂O₂, and the remaining H₂O₂ was determined (21, 22). We calculated that, after catalase treatment, a maximum of 1 nmol H₂O₂ could have been present in the volume (100 μl) injected for induction of sensitization. With the Ni(III) remaining after the degradation of H₂O₂, we performed the MEST. The ear-swelling response of mice primed with Ni/H₂O₂ plus catalase was comparable with that of mice primed with Ni/H₂O₂ (Fig. 4). In the case of addition of OCl⁻, the resulting suspension of Ni(IV) was precipitated by centrifugation so that excess OCl⁻ in the supernatant was removed, as described under Materials and Methods. The possibility remained, however, that some OCl⁻ was occluded in the black precipitate. For this purpose, in one test, instead of resuspending the precipitate in saline, it was dissolved in diluted HNO₃, and 0.3 M AgNO₃ was added. This did not lead to the characteristic white precipitate indicative of AgCl that would have been formed had OCl⁻ been present. Taken together, these results indicate that the induction of sensitization obtained with Ni(III) and Ni(IV), respectively, was due to the higher nickel oxidation states per se; adjuvanticity of the oxidants used played no, or at least no significant, role in this process.

Generation of CD4⁺ nickel-specific hybridoma clones
To prime mice to nickel, we decided to use nickel and carrier proteins, such as albumin, known to have nickel-binding capacity (30). In the first experiment, mice were immunized against Ni(II)Cl₂-preincubated MSA. After generation of T cell hybridoma clones from splenic T cells, only one clone of 136 clones tested exhibited specificity for Ni(II); i.e., it recognized Ni(II) in the presence of syngeneic spleen cells, irrespective of whether MSA was present or not (Table I). Thus, this clone was not specific for a
Ni(II)-MSA complex (data not shown). Next, we tested HSA as nickel-complexing agent because of its inherent antigenicity in mice: 59 of 360 hybridomas proved to be HSA specific, but no nickel-specific T cell hybridoma was identified upon immunization with Ni(II)Cl₂-preincubated HSA. We then chose Ni(IV)(OH)₂O for generation of T cell hybridomas. Following immunization against MSA preincubated with Ni(IV)(OH)₂O, 61 Ni(II)-specific T cell hybridomas were identified among 350 hybridomas screened; in addition, two T cell clones reacted against MSA alone and thus were autoreactive. In another immunization, we used bovine RNase A preincubated with Ni(IV)(OH)₂O; this protein, too, is known to bind Ni(II) (31). This time, 35 Ni(II)-specific T cell hybridomas were found among 384 hybridomas tested (Table I). Again, the nickel-specific T cell clones did not require the presence of the carrier protein used for immunization (Table I). Hence, for successful generation of Ni(II)-specific clones, not the kind of protein, but the oxidation state of the nickel compound used for immunization seems to be the crucial factor. Once they had been generated, however, our nickel-specific T cell hybridomas reacted equally well to Ni(II) and Ni(IV) (data not shown). It is noteworthy that the T cell hybridomas recognized nickel irrespective of whether the original protein used for priming was present during recall or not (Table I).

**Table I. Survey of the reactivity of CD4⁺ T cell hybridomas obtained after immunization against proteins complexed with either Ni(II) or Ni(IV)**

<table>
<thead>
<tr>
<th>Ag Used for Immunization</th>
<th>Number of T Cell Hybridomas Reacting Against</th>
<th>Total No. Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSA/Ni(II)</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>HSA/Ni(II)</td>
<td>59</td>
<td>360</td>
</tr>
<tr>
<td>MSA/Ni(IV)</td>
<td>2</td>
<td>61</td>
</tr>
<tr>
<td>RNase/Ni(IV)</td>
<td>162</td>
<td>350</td>
</tr>
</tbody>
</table>

*Clones reacted to Ni(II) and Ni(IV) irrespective of whether the protein used for immunization was present or not.

**FIGURE 4.** Sensitization by Ni(II) plus H₂O₂ is caused by Ni(III) per se and not by adjuvanticity of the H₂O₂. Groups of mice were injected intradermally into each flank with either 1 μmol Ni(II)Cl₂ plus 30 μmol H₂O₂, i.e., Ni(III), in saline, or with either of these substances alone. In one group of mice, after incubating Ni(II) with H₂O₂ for 1 h, catalase (3000 U/mg, i.e., 0.68 U/ml) was added to the mixture to delete excessive H₂O₂ that had not reacted with Ni(II). With the Ni(III) after removal of H₂O₂ we then performed the MEST. Ten days after priming, animals were challenged for recall by injection into each ear of either 0.2 μmol Ni(II)Cl₂ in saline. Data are expressed as mean ear-swelling response (× 10⁻⁶ mm) ± SD at 48 h after challenge (*, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; compared with the groups denoted by bars).

**FIGURE 5.** Characterization of nickel-specific CD4⁺ T cell hybridomas in the presence of nonfixed spleen cells as APC. After 24-h cell culture, supernatants were removed and assayed for IL-2 release. Means of triplicate values are shown. **A,** Reactivity of nickel-specific T cell hybridoma clones to increasing concentrations of Ni(II)Cl₂. Background proliferation of IL-2-dependent cells was 465 ± 95 cpm. **B,** Cross-reaction of nickel-specific clone F4 13A6 with CoCl₂. Reactivity to increasing concentrations of metal [Me(II)] salts, namely Ni(II)Cl₂ and CoCl₂, is shown. Background proliferation of IL-2-dependent cells was 1102 ± 677 cpm. **C,** Nickel-specific T cell hybridomas are MHC class II restricted. Five clones were cocultured with 100 μM Ni(II)Cl₂, either in the absence of mAb (open columns), or in the presence of anti-MHC class II mAb (black columns) or isotype-matched control mAb (hatched columns). Background proliferation of IL-2-dependent cells was 2490 ± 623 cpm.

**Dose-response relationships in responses of nickel-specific T cell hybridomas**

Responses of five different, randomly selected nickel-specific T cell hybridoma clones to increasing concentrations of Ni(II)Cl₂ are shown in Fig. 5A. Four of five clones reached their maximum IL-2 response at about 200 μM Ni(II), a concentration that is already toxic for Con A-stimulated T cells (4, 17) and reduced the proliferative capacity of T cell hybridoma clones (data not shown).
Limited cross-reactivity of T cell hybridoma clones with other metal ions

It has been shown that part of the human nickel-specific T cell hybridomas cross-react with copper, palladium, and/or cobalt (2, 4). To answer the question, whether some of the murine nickel-specific T cell hybridomas also show cross-reactivity, several transition metals, namely Au(I), Au(III), Co(II), Cu(II), Pt(II), and Pt(IV), were tested in specificity assays. Only one of 23 tested, F4 13A6, responded to one of these metal salts, namely Ni(II) (Fig. 5B). The stimulatory concentration range for Ni(II) and Co(II) was between 100 μM and 10 μM, with a maximum response at 100 μM. Even though the maximum response to Ni(II) exceeds that to Co(II) in the experiment shown, a preferential response of clone F4 13A6 to Ni(II) in comparison to Co(II) cannot be deduced from these results, because in other experiments the response to Co(II) was sometimes higher than that to Ni(II) (data not shown).

MHC dependence and TCR elements of nickel-specific T cell hybridoma clones

Human nickel-specific T cell clones presumably recognize Ni(II) chelate complexes formed with MHC molecules and unknown self peptides bound to them (4, 32). To test the MHC dependence of nickel-specific CD4+ T cell hybridoma clones also show cross-reactivity, several transition metals, namely Au(I), Au(III), Co(II), Cu(II), Pt(II), and Pt(IV), were tested in specificity assays. Only one clone of 23 tested, F4 13A6, responded to one of these metal salts, namely Co(II) (Fig. 5B). The stimulatory concentration range for Ni(II) and Co(II) was between 100 μM and 10 μM, with a maximum response at 100 μM. Even though the maximum response to Ni(II) exceeds that to Co(II) in the experiment shown, a preferential response of clone F4 13A6 to Ni(II) in comparison to Co(II) cannot be deduced from these results, because in other experiments the response to Co(II) was sometimes higher than that to Ni(II) (data not shown).

Response of nickel-specific T cell hybridomas to pretreated APC

It is conceivable that different nickel-specific CD4+ T cell hybridomas recognize different nickel-induced epitopes. Formation of these epitopes could, for instance, depend on the way nickel interacts with self protein, on how nickel-protein complexes are processed, or on the stability of nickel-peptide-MHC complexes that are presented by APC and subjected to repeated washing. The most straightforward possibility would be that T cells recognize Ni(II) complexes with dominant self peptides that are naturally presented by MHC class II molecules (32, 33). In this case, it might be expected that T cells also recognize fixed APC to which Ni(II) was added after the fixation. Alternatively, before peptide presentation by MHC II molecules Ni(II) could form chelate complexes with soluble or membrane-bound self proteins; the changes in protein conformation thus caused would alter processing of the respective proteins and lead to presentation of different Ni(II)-peptide complexes or of cryptic self peptides (17, 34). In this case, the T cells would recognize nickel-induced, processing-dependent epitopes that could not be formed by APC that were fixed before the addition of Ni(II).

To address these questions, we compared nonfixed and glutaraldehyde-fixed APC in hybridoma stimulation assays. Eighteen nickel-specific T cell hybridoma clones were cocultured with nonfixed or fixed APC in the absence or presence of Ni(II). One of these clones, F4 9D2, still responded when Ni(II) was added to previously fixed APC (Fig. 6A), whereas all other clones failed to do so (clone F4 14C4 shown as example). Consistent with this result, clone F4 D92 also was the only hybridoma clone of those tested that reacted with APC that were first pulsed with Ni(II) and thereafter fixed and washed (data not shown). Apparently, clone F4 9D2 recognizes a Ni(II)-peptide-MHC class II complex that is processing independent and involves a dominant self peptide that is not destroyed or deformed by glutaraldehyde fixation. For the other seventeen clones, exemplified by F4 14C4, it cannot be concluded with certainty, however, that they recognize processing-dependent epitopes induced by nickel. The reason for this is that the glutaraldehyde fixation could have destroyed or deformed the naturally presented self peptides with which Ni(II) prefers to complex. To decide between these possibilities, we stimulated 10 T cell hybridoma clones with nonfixed APC that had been pulsed for 24 h with Ni(II), or medium alone, and had then been washed. In the stimulation assay, either no Ni(II) or 100 μM Ni(II) was added. Data of three representative clones are shown in Fig. 6B. When Ni(II) was added during the stimulation assay, all clones responded, irrespective of whether Ni(II)-pulsed washed APC or medium-pulsed washed APC were used. This was expected because the APC were not fixed and were not subjected to washing after addition of Ni(II) in the assay. Only one clone, F4 9D2, also responded to Ni(II)-pulsed washed APC when no additional Ni(II) was provided in the assay; another clone, F4 13A6, showed a low, but reproducible, response under these conditions. The other eight clones, exemplified by F4 1D1, failed to respond to Ni(II)-pulsed washed APC and reacted only if the provision of Ni(II) was renewed in the stimulation assay. Apparently, this lack of response was due to the loss of Ni(II) from the recognized Ni(II)-peptide-MHC complexes during washing of Ni(II)-pulsed APC. Interestingly, clone F4 9D2, which also reacted to fixed APC treated with Ni(II) (Fig. 6A), recognized Ni(II)-pulsed washed APC without addition of Ni(II) in the assay (Fig. 6B); together with data shown in Fig. 5C, these findings suggest that clone F4 9D2 recognizes a stable complex formed by Ni(II) and a dominant self peptide presented by MHC II molecules.

Discussion

Here we demonstrated that mice, albeit reared and kept under conventional conditions, were readily sensitized to Ni(II) if one of the

<table>
<thead>
<tr>
<th>T Cell Hybridoma</th>
<th>Vα</th>
<th>Vβ</th>
<th>Pan α/β</th>
</tr>
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<tbody>
<tr>
<td>F4 8A2</td>
<td>8</td>
<td>8.1</td>
<td>+</td>
</tr>
<tr>
<td>F4 11C4</td>
<td>—</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>F4 11C2</td>
<td>—</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>F4 4C5</td>
<td>—</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>F4 13A6</td>
<td>—</td>
<td>14</td>
<td>+</td>
</tr>
<tr>
<td>F4 9D2</td>
<td>8</td>
<td>13</td>
<td>+</td>
</tr>
<tr>
<td>F4 3A6</td>
<td>—</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>F4 14C4</td>
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<td>—</td>
<td>+</td>
</tr>
<tr>
<td>F4 17C3</td>
<td>—</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>F4 8B5</td>
<td>—</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>F4 1D1</td>
<td>—</td>
<td>—</td>
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*+ denotes positive staining.

*The gene segment could not be identified with the set of mAbs used.

Table II. Staining of Ni-specific T cell hybridoma clones with FITC-conjugated, TCR-specific mAb

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following conditions was used for priming: 1) coadministration of Ni(II) with either CFA or an irritant, such as SDS or PMA; 2) administration of Ni(III), provided by mixing Ni(II)Cl₂ and H₂O₂ (12, 20); 3) administration of Ni(IV)(OH)₂O; or 4) systemic pre-

FIGURE 6. Comparison of T cell hybridoma responses to Ni(II) presented by fixed and nonfixed APC, respectively. A. Only clone F4 9D2 responded when Ni(II) was added to already fixed APC. Cells of F4 9D2 and a representative nonresponding clone (F4 14C4), respectively, were cocultured with unfixed (open columns) or glutaraldehyde-fixed (black columns) APC in the presence or absence of Ni(II)Cl₂. After 24 h of coculture, supernatants were removed and assayed for IL-2 release. Means of triplicate values are shown. Background proliferation of IL-2-dependent cells was 946 ± 175 cpm. B. Nonfixed APC (2.5 × 10⁶/ml) were cultured for 24 h in the absence (open bars) or presence of 100 μM Ni(II) (gray bars) and were then washed twice before they were added to T cell hybridoma clones; no Ni(II) was added during the stimulation assay. Alternatively, APC were cultured 24 h in the absence (hatched bars) or presence of Ni(II) (closed bars) and then washed. The Ni(II)-pulsed washed APC as well as 100 μM Ni(II) were then added to T cell hybridoma clones. Background proliferation of IL-2-dependent cells was 1373 ± 246 cpm.

What makes the four conditions specified above special that they allow for induction of sensitization to nickel? At the present time, we can only speculate on this. With regard to the first condition, it is not known which of the many different effects of CFA and irritants was responsible for the adjuvant effect seen in priming to Ni(II). A possible explanation could invoke the fact that SDS and CFA induce massive dermal infiltration by, and activation of, phagocytic cells (28, 29, 35), and this yields, among other, a high local production of reactive oxygen species, such as H₂O₂ and OCl⁻. Conceivably, these reactive oxygen species oxidized the poor sensitizer Ni(II) to Ni(III) and Ni(IV), where the latter two succeeded in priming the animals. This possibility is supported by the results obtained under the second and third condition specified above: Ni(III) was found capable of priming mice for nickel-specific recall responses in vivo and in vitro, whereas Ni(II) alone, or H₂O₂ alone, were not. As far as the third condition, administration of Ni(IV), is concerned, Ni(IV)(OH)₂O proved to be effective in two different experimental approaches. First, Ni(IV), but not Ni(II), primed mice for recall responses in the MEST. Second, when complexed with protein, Ni(IV), but not Ni(II), effectively primed CD4⁺ T cells so that we succeeded in generating nickel-specific hybridomas. It should be realized, however, that, once the T cells had been primed, the sole provision of Ni(II) sufficed for elicitation of secondary responses in all test systems used in vivo and in vitro.

Although we cannot exclude distinct mechanisms of adjuvan-
ticity operating in each of the conditions specified, it is conceivable that there is a final common pathway for their ability to induce hypersensitivity. This could be the activation and mobilization of nickel-laden dendritic cells (DC) to emigrate to the draining lymph node and there provide signal 1, i.e., nickel-induced neontigen, together with signal 2 to naive T cells. The activation of resident DCs is known to be inducible by nonspecific tissue lesion in their environment (36, 37). The superior capacity of Ni(III) and Ni(IV) for induction of sensitization presumably is due to their higher chemical reactivity and, hence, greater toxicity when compared with Ni(II). Thus, unlike Ni(II), Ni(III) and Ni(IV) could trigger the activation and mobilization of DCs by exerting a nonspecific local toxicity, a “danger” signal (38). Although CFA, PMA, SDS, and H₂O₂ per se are able to activate DC, it is conceivable that the mobilizing effect of all these agents is enhanced when combined with the greater chemical reactivity of nickel at higher oxidation states. This is supported by our results obtained from experiments for induction of nickel-specific T cell hybridomas. Here, CFA plus 0.15 μmol Ni(IV) proved to be more effective in sensitizing than CFA plus the same dose of Ni(II). Other than for the induction of T cell hybridomas, priming with Ni(II) admixed to CFA was successful in the MEST; there, however, the nickel dose used (1.0 μmol/mouse) was 6.5-fold higher than that used for induction of nickel-specific T cell hybridomas.

Obviously, Ni(II) can provide signal 1 but, when given alone, lacks the capacity for evoking a sufficient signal 2 for the priming of naive T cells. As evident from the fourth condition specified above, the lack of signal 2 could also be compensated for by systemic treatment with rIL-12. IL-12 appears to be critically involved in the induction of contact hypersensitivity, since blocking of IL-12 in vivo by injection of neutralizing Abs directed against IL-12 inhibits contact sensitization against haptens (26, 27). The current concept of the induction phase of sensitization implies that Langerhans cells migrate out of the skin to the regional lymph nodes. During this migration, they mature into effective APC with all the costimulatory molecules required for T cell priming. Macatonia et al. showed that DCs are a potent source of IL-12 and thereby direct the development of Th1 cells from naive CD4⁺ T cells (39). The dominant role of IL-12 during sensitiza-
tion is also indicated by the fact that IL-12 was identified as the first cytokine that is able to prevent and even break established hapten tolerance (27, 40, 41).

Theoretically, another possibility that could explain the greater immunogenicity of higher nickel oxidation states is that these might bind with greater affinity than Ni(II) and thus form more stable complexes with proteins and peptides; a quantitative difference in stability of protein complexes could result in increased
immunogenicity. Consistent with this possibility, the binding constants for Ni(III) to terpyridine are greater than for Ni(II) (42). In any event, however, a supposed greater stability of protein or peptide complexes with nickel at higher oxidation states did not result in formation of neoantigens that were so stable that in the elicitation of recall responses they would have required presence of nickel-protein complexes that were identical with those used for priming, e.g., bovine RNase complexed with Ni(IV).

What is the chemical basis of the oxidation of Ni(II) to the higher oxidation states of nickel, which, as we hypothesize, may take place in vivo? With regard to the formation of Ni(III), it has to be taken into account that, due to charge delocalization favored by ligands with high electron density, the standard electrode potential of Ni(II)/Ni(III) can be dramatically lowered when Ni(II) is complexed by peptides or proteins (43). This allows Ni(II)-peptide complexes to be oxidized to Ni(III)-peptide complexes by rather mild oxidants, such as molecular oxygen or H$_2$O$_2$ (20), in a Fenton-like reaction: Ni(II) + H$_2$O$_2$ → Ni(III) + OH$^-$ + OH$^-$ (44).

Both Ni(III) and the hydroxyl radical are chemically more reactive than their precursor molecules, Ni(II) and H$_2$O$_2$, respectively and, hence, should be more powerful elicitors of the danger signal (38). As far as production of Ni(IV) is concerned, this can be generated from Ni(II) by reaction with OCl$^-$ (13), a powerful oxidant that is formed from H$_2$O$_2$ by myeloperoxidase; both H$_2$O$_2$ and myeloperoxidase are released from activated neutrophils and monocytes into the microenvironment.

The present paper is the first to report the generation of nickel-specific murine T cell hybridomas. The nickel-specific T cell hybridoma clones could be restimulated not only by the higher nickel oxidation state used for priming, but also by Ni(II). In the latter respect, the results obtained by the study of hybridomas conform with those obtained from the MEST, the PLN assay, and the in vitro testing of human T cell clones specific for nickel (3, 4). The crucial difference between the higher oxidation states of nickel on the one hand side and Ni(II) on the other hand side seems to be their differential capacity for induction of costimulatory signals rather than a differential capacity for provision of signal 1. Since the requirements for costimulation, in particular DC mobilization, are lower in secondary than in primary immune responses, the defective capacity of Ni(II) for induction of costimulation needs not be disclosed when studying recall responses. Ni(II) on the other hand does induce ICAM and E-selectin on endothelial cells (45, 46) so that primed T cells are enabled to get to the site of challenge with Ni(II) in the mouse ear.

Unexpectedly, the presence of the proteins MSA and RNase, respectively, that was part of the Ni(IV)-protein complex used for priming was not required for eliciting recall responses by the nickel-specific T cell hybridomas. In all likelihood, the clones reacted to MHC-embedded peptides cleaved from unidentified self proteins that were altered by Ni(II) and Ni(IV), respectively, in one way or the other. Hence, none of the nickel-binding proteins used for priming acted like the classical protein carriers of covalently bound organic haptons, such as trinitrophenyl derivatives (47, 48), that provide the peptide part of the MHC-embedded hapten-peptide conjugate. Two different explanations may account for this lack of requirement of the original protein used for priming. First, the proteins used for priming to nickel merely served as vehicles conserving higher oxidation states of the metal; this was followed by ligand exchange and complex formation with unknown self proteins or self peptides, and it was these nickel complexes that actually primed naive T cells. Alternatively, some of the epitopes resulting from nickel complexing with the exogenous proteins used for priming were identical with those that resulted from the complexing of nickel ions with unidentified self proteins or peptides. If so, this would imply that the recognition of Ni(II) by the TCR is carrier independent to a certain degree, similarly as has been observed with some of the trinitrophenyl-specific T cell clones (47, 48) and some of the human nickel-specific T cell clones (49).

With respect to their MHC class II dependence, TCR diversity, limited cross-reactivity, and recognition of processing-dependent and -independent nickel epitopes, the murine CD4$^+$ nickel-specific T cell hybridomas reported here closely resemble the human nickel-specific T cell clones described in the literature (2, 4). As several of their nickel-specific CD4$^+$ clones also reacted to glutationaldehyde-fixed APC to which Ni(II) was added after the fixation, Weltzien and coworkers (4) concluded that these clones are processing independent, suggesting that they recognized nickel complexed with naturally presented self peptides. Likewise, one of our nickel-specific T cell clones, F4 9D2, recognized fixed APC to which Ni(II) was added after the fixation and, thus, presumably recognized naturally presented self peptides complexed with Ni(II). Moreover, this particular epitope proved to be relatively stable because it was not lost when nonfixed Ni(II)-pulsed APC presenting the epitope were subjected to repeated washing. In contrast, the epitope(s) recognized by most other T cell hybridomas tested was unstable by this criterion. The other part of the CD4$^+$ nickel-specific clones described by Weltzien et al. (4) was classified as processing dependent because they failed to respond to fixed APC plus Ni(II). Likewise, the majority of our T cell hybridomas failed to recognize fixed APC plus Ni(II) and, therefore, might be interpreted to be processing dependent. Processing dependence of Ni-induced epitopes implies that nickel interacts with soluble or membrane-bound proteins and leads to altered processing of the self proteins thus complexed and, hence, to presentation of either Ni(II)-complexed or cryptic self peptides (17, 34, 50).

This conclusion should be drawn with caution, however, since loss of T cell responsiveness in the presence of fixed APC and Ni(II) does not necessarily prove processing dependence. Fixation of APC with glutaraldehyde cross-links their surface proteins and could thereby alter the conformation of peptide-MHC complexes such that the APC, upon addition of nickel, can no longer form the same metal-peptide-MHC complexes as unfixed APC and, hence, need no longer be recognized by a given T cell clone. Although it is known that the oxidation state of heavy metals may be subject to change in vivo and that such changes may profoundly alter their biological function, little attention has been paid to this in allergology. An exception here is the oxidative conversion of chemically less reactive gold(I) into the highly reactive gold(III) intermediate (15, 18). As mentioned before, the bioxide of Ni(II) by H$_2$O$_2$ and OCl$^-$, respectively, parallels that of gold(I) in that in both cases the higher oxidation states of heavy metal proved to be much stronger inducers of sensitization than the lower ones (Refs. 14, 16, and 34, and results of the present paper). However, there also seems to be a difference between the two metals, and this concerns the reason why their lower oxidation states, gold(I) and Ni(II), are inadequate agents for T cell priming. In the case of gold, the difference between the lower and the higher oxidation state lies in its differential ability to generate neoantigens, or signal 1; neoantigen formation by gold(III) is due to oxidation and, hence, irreversible denaturation of protein (17, 34), something the lower oxidation state, gold(I), is unable to do. In contrast, the inability of the lower oxidation state of Ni, Ni(II), for induction of sensitization should be sought not in inadequate provision of signal 1, but in an inability to elicit effective signal 2 for T cell priming. The latter can be efficiently elicited by higher oxidation states of nickel, which presumably are generated under conditions such as
ENHANCED SENSITIZATION VIA NICKEL AT HIGHER OXIDATION STATES

injury, inflammatory skin disease, or concomitant exposure to irritating chemicals.

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