Persistent Immune Tolerance to Nickel and Chromium by Oral Administration Prior to Cutaneous Sensitization

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Oral administration of allergens, foreign proteins, or cell-bound antigens may induce systemic suppression of subsequent humoral and cell-mediated immune responses ("oral tolerance"). The induction of specific immune tolerance provides a potential strategy for treatment of T-cell–dependent immune diseases. Therefore, in depth studies into preconditions for optimal and persistent tolerance induction are mandatory. Here we report on such studies in a guinea pig model using the non–cross-reactive contact allergens nickel and chromium. Feeding per os of nickel sulfate or potassium dichromate did not trigger systemic T_{DTH}-effector functions. Instead, short feeding periods led to a dose-dependent, and metal-specific, suppression of subsequently induced allergic contact hypersensitivity. Administration of the allergens onto the oral mucosa was most effective in the induction of immune tolerance. When first sensitizing attempts were delayed until 1 year after feeding, the degree of unresponsiveness was reduced. In contrast, with cutaneous contacts starting shortly after the feeding period, tolerance was fully stable and undiminished for at least 2 years. Thus, in orally treated guinea pigs cutaneous contacts provide boosting tolerogenic signals, supporting the view that oral tolerance does not result from clonal deletion but from active antigen-specific immunosuppression. Indeed, unresponsiveness to cutaneous immunization could be transferred by lymphoid cells from fed guinea pigs in a metal-specific way. *J Invest Dermatol 99:608–616, 1992*

Specific down-regulation of undesired T-cell immune functions would be of great importance in the combat against allograft rejection, autoimmune, and allergic diseases. Currently, the majority of clinical approaches for treatment of these diseases is with broadly immunosuppressive drugs that leave the patients vulnerable to opportunistic infections. A potential strategy to prevent T-cell–dependent immune diseases is the induction of a condition of selective inability to mount systemic immune responses to an antigen. A promising way to induce such specific immunologic tolerance is by intragastric administration of the antigen. The phenomenon of "oral tolerance" was first demonstrated for the (T-cell–dependent) humoral immune response to ovalbumin [1] and later also for cellular immune responses (delayed-type hypersensitivity, DTH) to the haptenic chemical dinitrochlorobenzene [2]. Since then, it has been shown that systemic humoral responses and systemic cell-mediated immunity (CMI) responses can be suppressed after gavage in immunologically naive animals with various T-dependent antigens, such as soluble protein antigens (reviewed in [3,4]), contact sensitizing agents [5–7], red blood cell–bound antigens (reviewed in [8–10]) and inactivated bacteria or viruses [11,12]. Interestingly, it appeared to be very difficult to interfere with secretory immunoglobulin (IgA) responses, which have been ascribed a role in inhibiting intestinal uptake of intact antigen [13–15].

The digestive tract is daily exposed to a great variety of dietary antigens and therefore has to meet conflicting requirements. On one hand, effective immune responses are to be mounted against potential pathogens/allergens. On the other hand, these immune responses should not cause undue damage to the alimentary tract. It has therefore been hypothesized that the phenomenon of oral tolerance, together with an unimpaired mucosal IgA response, may represent an important pathway to prevent enteropathies [3,4,16]. Because only recently the potential therapeutic power of oral tolerance induction has begun to be appreciated, the mechanism(s) underlying oral tolerance are still poorly understood [17]. Antigen-specific suppressor T cells [3,18], antigen-antibody complexes [19], anti-idiotypic antibodies [20], as well as soluble suppressor factors [21] have all been suggested to play a role in oral tolerance. Oral tolerance may affect the humoral or cellular immune response, with the other being normal or enhanced ("split tolerance") [22–25]. The intimate relationships between cell-mediated immune effector functions and humoral immune responses adds to the complexity of the issue [3].

Recently we developed a guinea pig allergic contact hypersensi-

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Abbreviations:
ACH: allergic contact hypersensitivity
CMI: cell-mediated immunity
DMSO: dimethyl sulfoxide
DTH: delayed-type hypersensitivity
FCA: Freund's complete adjuvant containing Mycobacterium butyricum
IgA: immunoglobulin A
SEM: standard error of the mean

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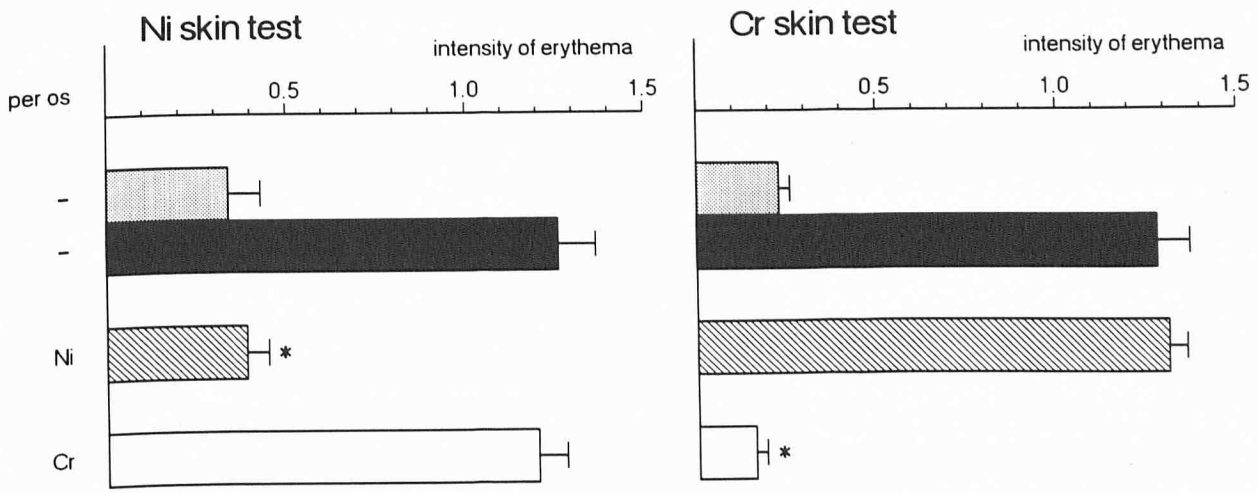

Figure 1. Antigen specificity of oral tolerance. Groups of guinea pigs were fed with 30 mg $NiSO_4$ or 30 mg $K_2Cr_2O_7$ in apple sauce weekly for 6 weeks. Sensitization to both nickel and chromium was attempted 2 weeks after the feeding period. Three weeks after sensitization the guinea pigs were challenged epicutaneously to reveal eventual development of nickel *(left)* and chromium *(right)* hypersensitivity. Data are expressed as mean intensity of erythema ± SEM of seven guinea pigs per group at 48 h after challenge. *Dotted bars,* animals immunized with FCA only; *black bars,* animals sensitized to both nickel and chromium; *hatched bars,* animals fed with $NiSO_4$ and sensitized to both metals; *open bars,* animals fed with $K_2Cr_2O_7$ and sensitized to both metals. *$p < 0.001$ as compared to positive control group.

tivity (ACH) model for concomitant nickel and chromium sensitization [26]. Nickel and chromium are clinically relevant T-cell allergens that are thought to modify major histocompatibility complex-bound peptides resulting in metal-specific T-cell activation [27]. Although nickel and chromium are closely located within the periodic table of the elements, both animal [26,28] and clinical [29,30] studies excluded the existence of crossreactivities. Simultaneous use of both metal allergens, therefore, allows for detailed specificity studies in further developing optimal oral tolerogenic procedures. Moreover, because no systemic humoral antibody responses are generated in nickel or chromium hypersensitivity ([28], Scheper, unpublished data), oral tolerance induction procedures can be evaluated strictly in terms of CMI suppression. An advantage of guinea pig ACH models, as compared to mouse models, is that cutaneous sensitization is highly persistent, thus closely resembling the situation in humans [31]. The present study was designed to explore several features of oral tolerance induction, including dose, frequency, timing, and allergen routing. Moreover, metal specificity, transfer of tolerance with lymphocytes, and the persistence of orally induced tolerance, in relationship to timing of tolerogenic and sensitizing procedures, were studied.

MATERIALS AND METHODS

Animals Female albino guinea pigs of the outbred Dunkin Hartley strain (Harlan/TNO, Zeist, The Netherlands) were used, unless mentioned otherwise. In some experiments female outbred Himalayan strain (Harlan/TNO) or inbred strain 13 guinea pigs from our own breeding stock (Klinisch Dierexperimenteel Laboratorium, Vrije Universiteit, Amsterdam) were used. Animals were housed in standard macrolon cages, with free access to water and pelleted food. At the beginning of each experiment, after acclimatization for 1–2 weeks, the age of the animals was 6–8 weeks unless mentioned otherwise.

Oral Administration of Nickel and Chromium Groups of guinea pigs (consisting of five to eight animals each) were fed with different amounts of the metal salts nickel sulfate ($NiSO_4 \cdot 6H_2O$, Merck, Darmstadt, Germany) or potassium dichromate ($K_2Cr_2O_7$, Merck) in apple sauce. The apple-sauce mixtures were prepared shortly before administration. Each time, 1 ml of a mixture was given by means of a blunt needle. Because the taste of the metal salts was camouflaged by the apple sauce, animals were cooperative in swallowing the putative tolerogenic mixtures. Guinea pigs were fed for periods from 1 to 6 weeks. In some experiments induction of unresponsiveness was performed by intragastric feeding or by application onto the oral mucosa. Intragastric and mucosal (oral cavity) administrations were performed under light anesthesia with, respectively, 0.5 and 1 μl/g body weight of a 4:3 mixture of Aescoket (Aesculaap Boxtel, The Netherlands) and Rompun (Bayer, Leverkusen, Germany) by intramuscular injection. For intragastric feeding the metal salts were dissolved in apple sauce and administered by a plastic tube. For "oro-mucosal" application metal salts were dissolved in distilled water and used for preparation of a mucosal ointment, Unguentum Hypromelosum (10% distilled water). Nickel or chromium salt-containing ointments were applicated to the oral mucosa with a small spatula. After 1 h the ointment was removed by means of the spatula and rinsing with warm water.

Concomitant Induction of Nickel and Chromium Contact Hypersensitivity The development of a nickel and chromium double-sensitization model in the guinea pig is described elsewhere [26]. Briefly, the animals received four intradermal injections with 2 mg $K_2Cr_2O_7$ per ml of a 1:1 mixture of saline and Freund's complete adjuvant containing *Mycobacterium butyricum* (FCA; Difco Laboratories, Detroit, MI). They received 2 × 0.1 ml into the clipped thigh site of the dorsal skin, and 2 × 0.05 ml into the pinnae of the ears. In addition, two injections with 0.1 ml FCA emulsion (without metal salt) were given into the clipped shoulder site of the dorsal skin. After 24 h, 4 × 0.05 ml of 3 mg $NiSO_4$/ml saline were injected into the four adjuvant sites on the dorsal skin. Thus, the animals received total doses of 0.6 mg $K_2Cr_2O_7$, 0.6 mg $NiSO_4$, and 0.5 ml FCA emulsion into six injection sites. Control animals received emulsified FCA only.

Skin Tests Skin tests were performed and evaluated as described elsewhere [26]. Briefly, animals were challenged on clipped and freshly depilated dorsal midflank skin sites. The depilatory cream used (Biodermal) was obtained from Dr. H. Schreuder's Laboratory (Baarn, The Netherlands). One week after oral administration procedures animals were routinely skin tested by intradermal injection of 25 μg $NiSO_4$ in 0.1 ml saline, and 25 μg $K_2Cr_2O_7$ in 0.1 ml saline. This test would reveal a possible sensitizing effect of the oral administration. Sensitizing attempts were made from 1 week later onwards. Two weeks after any sensitizing attempts animals were re-challenged intradermally to reveal eventual development of nickel and chromium hypersensitivity. Routinely, guinea pigs received epicutaneous skin tests on contralateral flanks 1 week after

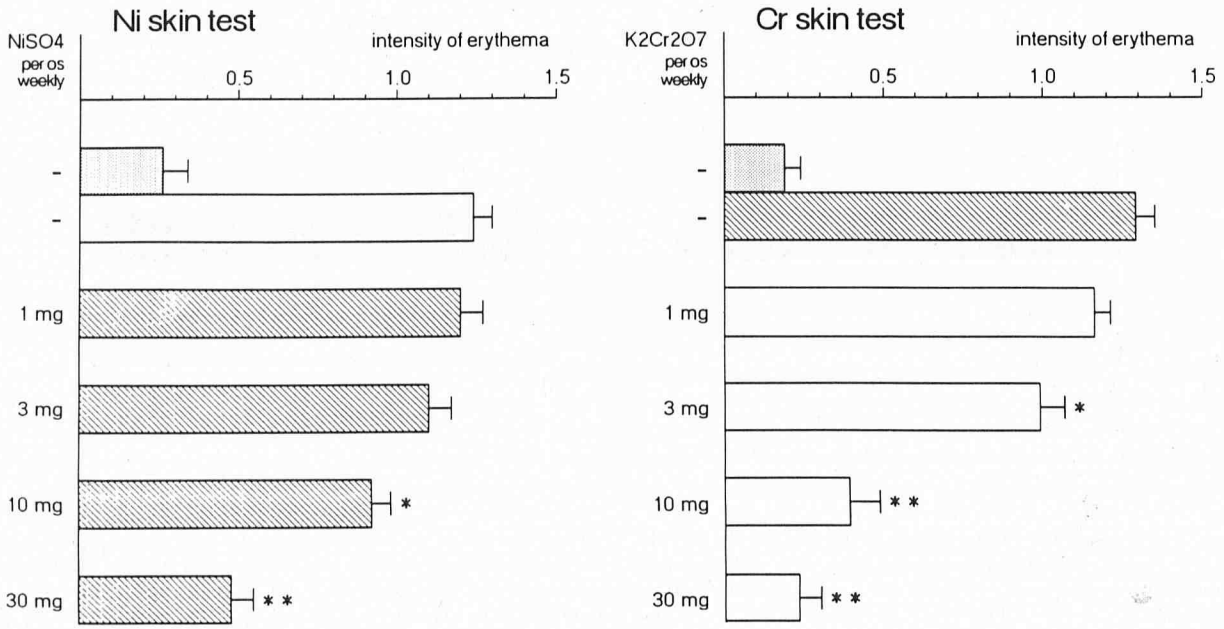

Figure 2. Dose dependency of oral tolerance shown by varying the dose per feed. Groups of guinea pigs were fed weekly for 3 weeks wth 1, 3, 10, or 30 mg $NiSO_4$ or $K_2Cr_2O_7$ in apple sauce. Sensitization to both nickel and chromium was attempted 2 weeks after the feeding period. Three weeks after sensitization the guinea pigs were challenged epicutaneously to reveal eventual development of nickel *(left)* and chromium *(right)* hypersensitivity. Data are expressed as mean intensity of erythema (±SEM) of six to seven guinea pigs per group at 48 h after challenge. *Dotted bars,* animals immunized with FCA only; *hatched bars,* animals fed with $NiSO_4$ and sensitized to both metals; *open bars,* animals fed with $K_2Cr_2O_7$ and sensitized to both metals. *$p < 0.01$, **$p < 0.005$, as compared to positive control group (animals fed with the other metal).

intracutaneous skin testing. Twenty microliters of 10% $NiSO_4$ and of 0.5% $K_2Cr_2O_7$ in 40% dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) in distilled water were applied onto dorsal flank skin on a circular area (diameter of 1 inch) and dried with an electric hair drier. Skin reactions were evaluated at 24, 48, and 72 h. The intensity of erythema was scored according to the following scheme: equivocal or no reaction = 0; pink, spots = 0.5; red, confluent = 1.0; intense red, swollen = 2.0. Scores given to individual animals represented the mean of at least two independent observers. For intradermal reactions also the diameter of erythema was recorded. In *Results,* 48-h reactions from the final epicutaneous skin test are shown, unless only the intradermal skin test was performed. For the intradermal test, 48-h skin reactions are expressed as a product score of diameter and intensity of erythema.

Transfer of Orally Induced Tolerance for Nickel or Chromium One week after finishing a tolerizing feeding procedure, cervical, paratracheal, and mesenteric lymph nodes and spleens were isolated in medium (RPMI 1640 containing 25 mM Hepes buffer with L-glutamine, Gibco, Paisley, Scotland, UK) with 2% fetal calf serum, minced with scissors, and squeezed through a nylon gauze filter to give single cell suspensions. Pooled lymphoid cells were washed, counted, and suspended in the desired volume of phosphate-buffered saline. Recipient guinea pigs received donor equivalents of cells (4×10^8) in 2 ml of phosphate-buffered saline into an ear vein and were immunized with both nickel and chromium within 16 h. Intradermal and epicutaneous challenges were performed 14 and 21 d later, respectively.

Statistical Analysis The results are expressed as the arythmetic mean ± SEM of values per group (at least five animals). Comparisons between groups were made using the non-parametric Wilcoxon test. The level of significance was set at $p < 0.05$.

RESULTS

Oral Administration of Nickel Sulphate or Potassium Bichromate Specifically Suppress Subsequent Development of ACH To induce oral tolerance, groups of guinea pigs were fed once a week for 6 weeks with 30 mg of either nickel sulfate or potassium bichromate, whereas control groups received vehicle (apple sauce) only. Intradermal skin tests performed 1 week after the last feeding showed that feeding never induced any detectable degree of sensitization (data not shown). Subsequently, it was attempted to sensitize both metal-fed groups and a control group to both nickel and chromium. All groups, including a control group sensitized with adjuvant only, were again skin tested 2 and 3 weeks later. Both intradermal and epicutaneous skin tests showed that feeding of either of the metal salts resulted in immunologically specific unresponsiveness. Nickel-sulfate feeding had induced strong unresponsiveness (95% suppression) to subsequent nickel sensitization, whereas the bichromate fed animals were completely refractory to chromium sensitization (106% suppression). Chromium sensitization was not affected in nickel-fed guinea pigs and vice versa (Fig 1). Tolerance induction by feeding was obtained in outbred Duncan Hartley and outbred Himalayan guinea pigs, as well as in inbred strain 13 guinea pigs. No differences in dose-response relationships or degree of unresponsiveness to subsequent cutaneous sensitization were observed (data not shown).

Dose Dependence of Oral Tolerance Induction Next, dose dependence of oral tolerance induction was studied for both metal salts. First we varied the dose per feed at a fixed period of weekly feedings. In these experiments, groups of guinea pigs received weekly doses of nickel sulfate or bichromate from 30 mg down to 1 mg for a total period of 3 weeks. Again, feeding with the highest oral doses for potassium dichromate led to strong tolerance (93% suppression) in all animals (Fig 2, *right*) whereas for nickel sulfate the degree of tolerance was slightly lower (78% suppression; Fig 2, *left*). As expected, with lower doses of the metals a dose-dependent reduction in the degree of tolerance was observed. Dose dependency of oral tolerance induction was also demonstrated by varying the period of weekly feedings, at fixed weekly oral doses. By extending the period of weekly feeding, stronger immune tolerance to nickel or chromium ACH could be obtained (Fig 3).

Frequency Dependence of Oral Tolerance Induction We then investigated the frequency dependence of oral tolerance induction, that is, whether, given a total dose and time span, the number

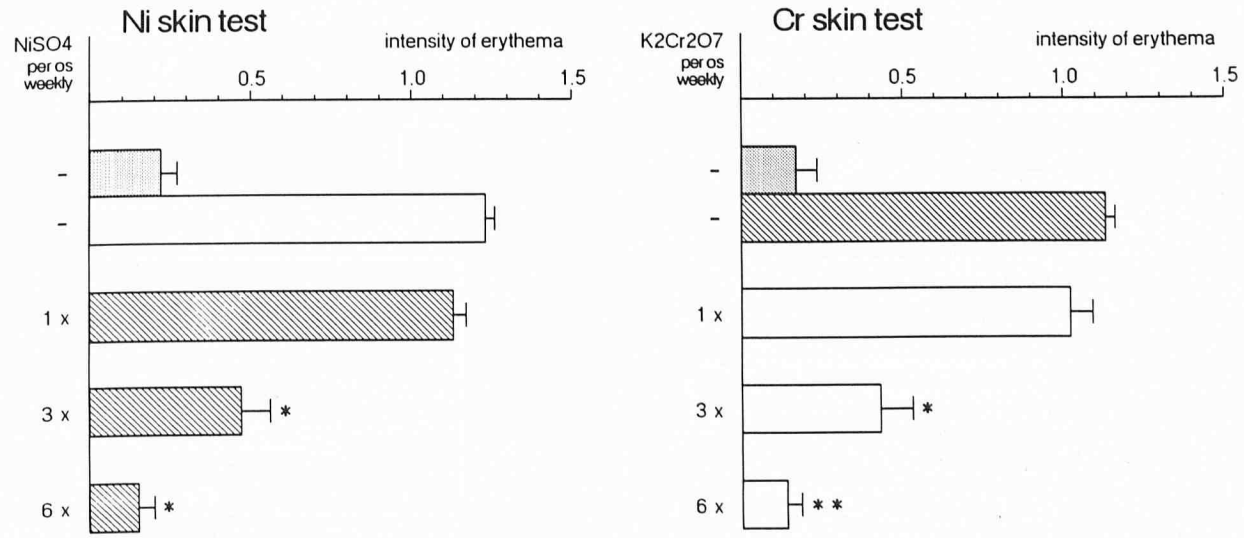

Figure 3. Dose dependence of oral tolerance shown by varying the number of weekly feedings. Groups of guinea pigs were fed weekly for 1, 2, or 3 weeks with fixed doses of $NiSO_4$ (30 mg) or $K_2Cr_2O_7$ (10 mg) in apple sauce. Sensitization to both nickel and chromium was attempted 2 weeks after the feeding period. Three weeks after sensitization the guinea pigs were challenged epicutaneously to reveal eventual development of nickel *(left)* and chromium *(right)* hypersensitivity. Data are expressed as mean intensity of erythema (±SEM) of six to seven guinea pigs per group at 48 h after challenge. *Dotted bars,* animals immunized with FCA only; *hatched bars,* animals fed with $NiSO_4$ and sensitized to both metals; *open bars,* animals fed with $K_2Cr_2O_7$ and sensitized to both metals. $^*p < 0.005$, $^{**}p < 0.001$, as compared to positive control group (animals fed with the other metal salt).

of feeds would affect the degree of unresponsiveness. Therefore, groups of guinea pigs were fed with a total dose of 30 or 90 mg of nickel sulfate or potassium bichromate. The 30-mg dose was administered once (28 d before sensitization), in doses of 10 mg weekly for 3 weeks (28, 21, and 14 d before sensitization, respectively), or in doses of 3.3 mg three times weekly for 3 weeks. The 90-mg dose was administered in doses of 30 mg weekly for 3 weeks or in doses of 10 mg three times weekly for 3 weeks. A single feed of 90 mg was not included to avoid unacceptable toxicity (Fig 4). The results of this experiment indicate that, rather than a high frequency of feeding, higher oral doses per feed induce strongest tolerance. The data also confirm that ACH for chromium is more readily suppressed (strong tolerance even after a single feed of 30 mg) than for nickel. In other experiments with both nickel and chromium we

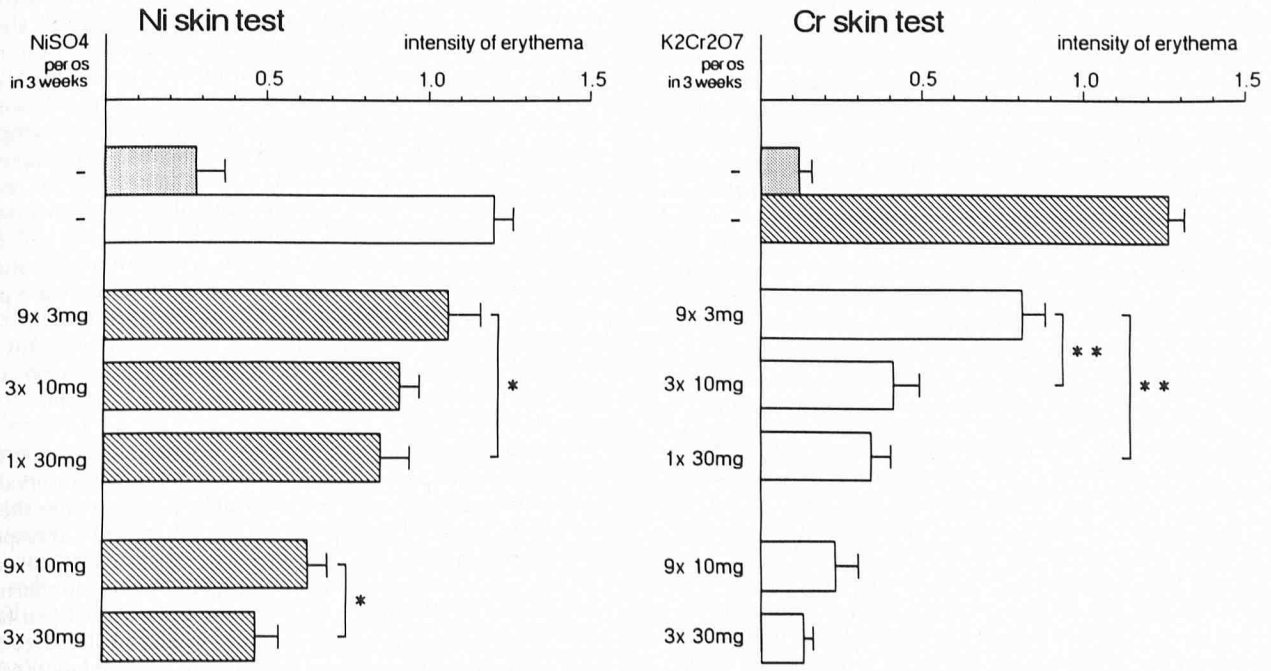

Figure 4. Frequency dependence of oral tolerance induction. Groups of guinea pigs were fed with fixed total doses (30 or 90 mg) of $NiSO_4$ or $K_2Cr_2O_7$ divided over 1, 3, or 9 feedings given in a 3-week time span. Sensitization to both nickel and chromium was attempted 2 weeks after the feeding period. Three weeks after sensitization the guinea pigs were challenged epicutaneously to reveal eventual development of nickel *(left)* and chromium *(right)* hypersensitivity. Data are expressed as mean intensity of erythema (±SEM) of six to seven guinea pigs per group at 48 h after challenge. *Dotted bars,* animals immunized with FCA only; *hatched bars,* animals fed with $NiSO_4$ and sensitized to both metals; *open bars,* animals fed with $K_2Cr_2O_7$ and sensitized to both metals. $^*p < 0.05$, $^{**}p < 0.01$.

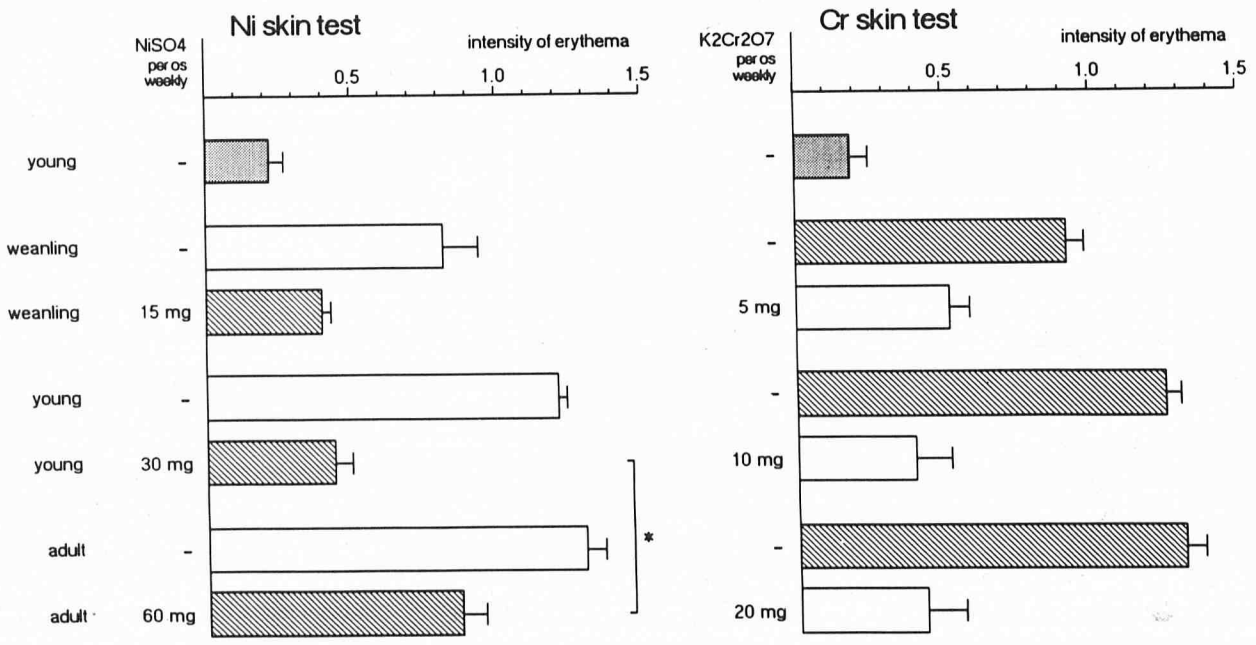

Figure 5. Age dependence of oral tolerance induction. Groups of guinea pigs with age 2–3 weeks (weanlings), approximately 8 weeks (young animals; routinely used in previous experiments), and 16–20 weeks (adults) were fed weekly for 3 weeks with either 75 mg/kg $NiSO_4$ (15, 30, and 60 mg, respectively) or 25 mg/kg $K_2Cr_2O_7$ (5, 10, and 20 mg, respectively). Sensitization to both nickel and chromium was attempted 2 weeks after the feeding period. Three weeks after sensitization the guinea pigs were challenged epicutaneously to reveal eventual development of nickel *(left)* and chromium *(right)* hypersensitivity. Data are expressed as mean intensity of erythema (±SEM) of six to eight guinea pigs per group at 48 h after challenge. *Dotted bars,* young animals immunized with FCA only; *hatched bars,* animals fed with $NiSO_4$ and subsequently sensitized to both metals; *open bars,* animals fed with $K_2Cr_2O_7$ and subsequently sensitized to both metals. *$p < 0.05$.

observed that the degree of specific immune tolerance was slightly lower when the single feed was administered at 14 d, instead of 28 d, before sensitization (data not shown).

Age Dependence of Oral Tolerance Induction We then studied oral tolerance induction in relation to age. For this purpose, suboptimal regimens for oral tolerance induction were selected: 30 mg nickel sulfate (75 mg/kg) or 10 mg potassium dichromate (25 mg/kg) at weekly intervals for 3 weeks. Three age groups were used: young animals (approximately 8 weeks of age), as had been used routinely in previous experiments, weanling (2–3 weeks of age), and adult (approximately 16–20 weeks of age) guinea pigs. Cutaneous immunizing attempts were made 4 weeks after the start of feeding. Both intradermal and epicutaneous skin tests revealed that the youngest (weanling) groups had not yet developed the degree of ACH observed in the older groups (Fig 5). This resulted in a slightly lower percentage suppression in the weanling chromium group after oral tolerance induction (55% versus 80%; not significant). Nevertheless, strong tolerance induction could be achieved in all age groups, although adult guinea pigs were significantly less susceptible to nickel tolerization (40% versus 78%; $p < 0.05$).

Tolerance Induction via Different Sections of the Alimentary Tract To study whether the oral mucosa plays a crucial role in tolerance induction we administered metal salts directly onto the oral mucosa as compared to intragastric administration. For comparison, groups tolerized by feeding per os were included. Suboptimal doses of 15 mg nickel sulfate or 10 mg potassium dichromate were used, at weekly intervals for 3 weeks. Intradermal skin tests performed 1 week after these treatments confirmed that none of the experimental groups exhibited any degree of sensitization. Subsequently, cutaneous immunization was attempted and resulting hypersensitivity reactions are shown in Fig 6. Intragastric administration induced a slightly lower degree of tolerance for both allergens, as compared to oral administration by feeding. In contrast, applications of the metal salts on the oral mucosa for only 1 hour at weekly intervals for three weeks resulted in strong unresponsiveness (for nickel, $p < 0.02$, as compared to feeding per os).

Transfer of Orally Induced Immune Tolerance by Lymphoid Cells We next questioned whether orally induced tolerance in this guinea pig model is due to active suppression and, therefore, might be transferred with lymphoid cells. Groups of donor inbred strain 13 guinea pigs were tolerized by feeding 30 mg $NiSO_4$ or 30 mg $K_2Cr_2O_7$ weekly for 6 weeks. For each allergen donor equivalents of pooled cervical, paratracheal, and mesenteric lymph node cells and spleen cells were injected intravenously into naive recipients (4×10^8 cells per recipient). Sixteen hours later all recipients were immunized to both allergens, and nickel and chromium hypersensitivities were assayed 2 (intradermal skin tests) and 3 weeks (epicutaneous skin tests) later. The results from these tests show that development of ACH to both nickel and chromium were partially prevented (Fig 7; for nickel $p < 0.05$ and for chromium $p < 0.02$). Because recipients of lymphoid cells from nickel-tolerant donors were used as positive controls for chromium sensitization, and vice versa, the (partial) immunotolerance was metal specific.

Persistence of Unresponsiveness Figure 8 shows that feeding with 30 mg $NiSO_4$ or 30 mg $K_2Cr_2O_7$ at weekly intervals for 6 weeks, followed by an attempt to sensitize (2 weeks after the period of feeding), induced a long-lasting state of specific unresponsiveness. Tolerance could not be broken by a repeated attempt to sensitize (25 weeks after the first attempt). Repeated intradermal and epicutaneous challenges up to 2 years after the initial oral feedings showed the persistent nature of orally induced tolerance. In contrast, when the first sensitizing attempt was made as late as 60 weeks after oral tolerogenic treatment, both the intradermal skin tests (2 weeks after sensitization; Fig 9) and the epicutanous skin tests revealed that considerably less resistence to sensitization had persisted in these animals in the absence of sensitizing attempts early (2 weeks) after oral treatment.

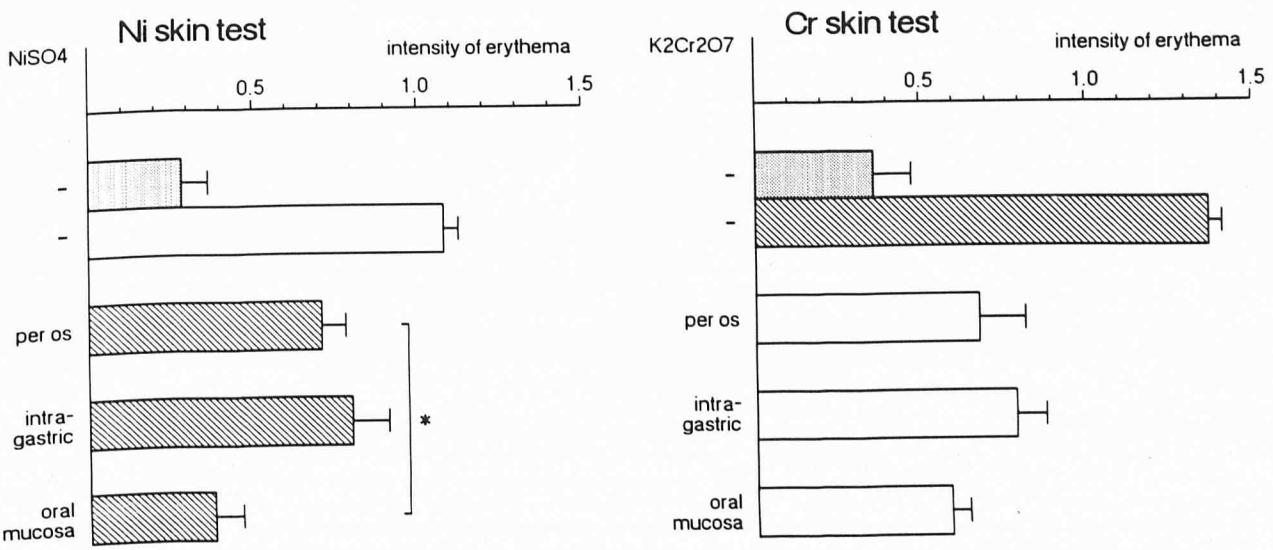

Figure 6. Tolerance induction via different sections of the alimentary tract. Groups of guinea pigs received 15 mg $NiSO_4$ or 10 mg $K_2Cr_2O_7$ either onto the oral mucosa, intragastrically, or by feeding per os, weekly for 3 weeks. For intragastric administrations and for feedings per os apple sauce was used as solvent. For mucosal administrations the salts were solved in an ointment (Unguentum Hypromelosum, 10% aqua dest.) that was applicated onto the oral mucosa of anesthesized animals. After one hour the ointment was removed. Sensitization to both nickel and chromium was attempted 2 weeks after the oral administration period. Three weeks after sensitization the guinea pigs were challenged epicutaneously to reveal eventual development of nickel *(left)* and chromium *(right)* hypersensitivity. Data are expressed as mean intensity of erythema (±SEM) of 5–8 guinea pigs per group at 48 h after challenge. *Dotted bars,* animals immunized with FCA only; *hatched bars,* animals orally treated with $NiSO_4$ and sensitized to both metals; *open bars,* animals orally treated with $K_2Cr_2O_7$ and sensitized to both metals. *$p < 0.02$ as compared to feeding per os.

DISCUSSION

Certain routes of antigen contact favor tolerance induction with one of the more classic routes being oral administration [3]. Because oral tolerance studies generally focus on suppression of subsequent parenteral immunization, potential development of effector T-cell function after oral administration has only received a little attention. In the present study all animals were routinely skin tested before any sensitizing attempts were made. One first important point to emerge from these studies, therefore, is that no single animal ever developed detectable skin hypersensitivity to either nickel or chromium after any of the oral feeding protocols tested. Thus, increasing the allergen dose, frequency, or period of feeding, intragastric feeding, or application onto the oral mucosa, for either of the metal allergens, never induced effector T-cell function. These findings favor the view that oral contacts with putative antigens do not induce CMI [13,16,32]. Major exceptions to this rule may, however, be created by the use of T-independent antigens [33], live infectious agents, or allogeneic cells [13,34–36], or the incorporation of strong adjuvants such as Cholera toxin or Iscoms [37,38]. With nickel and chromium the lack of induction of effector T-cell function after oral administration cannot simply be due to a low degree of allergenicity, because increased skin sensitivity to both metal allergens could be readily detected in naive guinea pigs after just one epicutaneous skin test.* Lack of detectable contact hypersensitivity could, therefore, theoretically result from three remaining possibilities. First, upon oral feeding the metal allergens would fail to be absorbed through the gastrointestinal mucosal cells and not come into contact with immune competent cells. This is apparently not the case, because feeding these metal allergens to previously sensitized animals induces profound, albeit transient, desensitization.* Moreover, in the mucosal tissues lining the gastrointestinal tract all cellular elements required for induction of cellular immune responses are present [39]. The alternative possibilities are that metal allergens reaching the circulation from the alimentary tract inactivate and/or eliminate precursor–effector T cells ("clonal deletion"), or induce active suppression ("immune tolerance"). Evidence for the latter mechanism was obtained here by extended time-course studies, the finding that a state of tolerance is not broken, but rather strenghtened by regularly sensitizing signals, and by lymphoid transfer studies.

In preliminary studies we noticed that feeding of pelleted food containing nickel and chromium could partly prevent subsequent sensitization to nickel or chromium [40]. Subsequently, oral tolerance to chromium was found to be more effectively induced with hexavalent than with trivalent chromium or with metal powder [41]. Using a recently developed guinea pig model for the induction of strong and reproducible ACH not only to chromium but also to nickel [26], we were able to study several features of orally induced T-cell tolerance in detail. From the results it is clear that nickel and chromium are excellent allergens to confirm the specificity of oral tolerance. Furthermore, in a dose-dependent way, complete unresponsiveness for systemic ACH/DTH could be achieved by controlled feeding of nickel or chromium salts during a limited period of time. At a given total dose, oral tolerance was stronger when the dose per feed was higher, indicating that it should be possible to achieve complete tolerance with a single high oral antigen dose. Indeed, for chromium almost complete tolerance was achieved by a single oral administration of 30 mg chromium salt (Fig 4). For nickel, however, this dose still was insufficient for the induction of full tolerance, whereas higher doses caused undue toxicity. Importantly, however, complete tolerance could also be achieved, even for nickel, by extending the period of (weekly) feedings with suboptimal tolerogenic doses (Fig 3). It has been well established that repeated skin contacts with contact sensitizing agents can augment the degree of ACH. The present data show that tolerogenic signals provide an essentially similar cumulative effect on the degree of immune tolerance. The clinical relevance of this notion was recently demonstrated by the finding that orthodontic-brace treatment can have a significant tolerogenic effect [42]. Although dental devices may release only low amounts of nickel, the frequency of nickel

* van Hoogstraten IMW, von Blomberg BME, Boden D, Kraal G, Scheper RJ: Non-sensitizing epicutaneous skin contacts prevent subsequent induction of immune tolerance (unpublished).

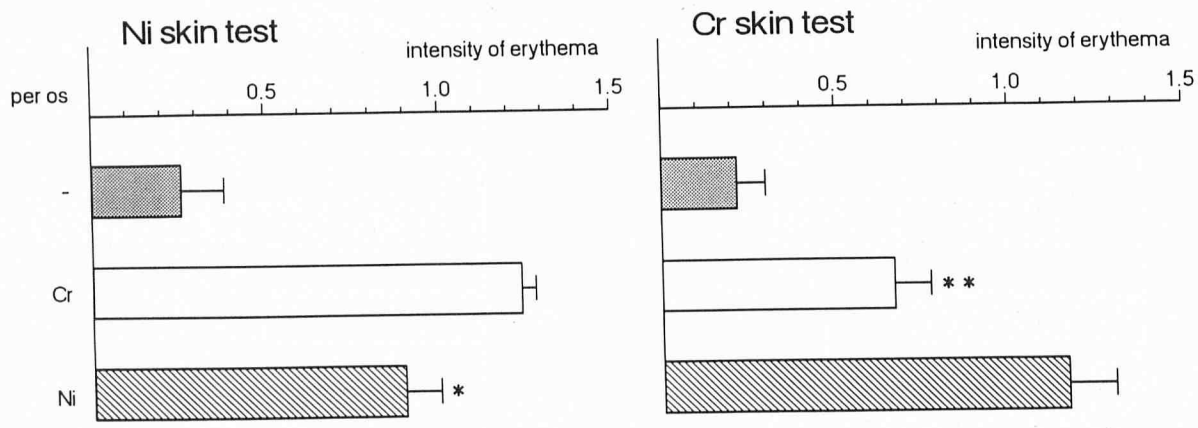

Figure 7. Adoptive transfer of oral tolerance. Recipients were injected intravenously with 4×10^8 cells from donors that had been fed weekly with either 30 mg $NiSO_4$ or 30 mg $K_2Cr_2O_7$ for 6 weeks. Feeding had been stopped 1 week before transfer. Cell pools were derived from spleen and cervical, paratracheal, and mesenteric lymph nodes. Sensitization to both nickel and chromium was attempted 16 h after transfer. Three weeks after sensitization the guinea pigs were challenged epicutaneously to reveal eventual development of nickel *(left)* and chromium *(right)* hypersensitivity. Data are expressed as mean intensity of erythema (±SEM) of five guinea pigs per group at 48 h after challenge. *Dotted bars,* animals immunized with FCA only; *hatched bars,* recipients injected with cells from donors fed with $NiSO_4$, and subsequently sensitized to both metals: *open bars,* recipients injected with cells from donors fed with $K_2Cr_2O_7$ and subsequently sensitized to both metals. $^*p < 0.05$, $^{**}p < 0.02$ as compared to transfer of cells from animals fed with the other metal salt.

allergy was significantly reduced in patients who had received orthodontic treatment at an early age, prior to possible sensitization.

Long-lasting persistence of orally induced tolerance has been noted for protein antigens in a mouse model [24]. The clinical relevance of such data is, however, questionable because the threshold for triggering suppressor cell functions in this species appears to be very low. This view is exemplified by data from contact sensitization studies showing that with higher doses of allergens applied onto the skin, mice readily develop allergen-specific suppressor cells [43]. Similar bell-shaped dose-response curves are not observed in the guinea pig, a species that is now known not to belong to the rodent genus [44]. Moreover, the rapid waning of ACH in mice, even with optimally sensitizing doses of allergens, is also thought to result from the development of immunoregulatory suppressor cells [43,45–47]. In contrast, ACH is very persistent in guinea pigs, like in humans [31,48]. Despite the paucity of immunologic reagents in the guinea pig, also immunohistologic features stress the relevance of guinea pig models for clinical ACH [31,49]. It was important, therefore, to note that in guinea pigs immune tolerance to nickel and chromium could still be detected 1 year after a short period of feeding (Fig 9). Equally important was the finding that oral tolerance could be boosted by skin contacts with the allergens, known to activate effector T-cell function in immunologically naive animals (Fig 8). If the state of tolerance would result from clonal deletion, such skin contacts would conceivably have no effect, or possibly contribute to the regeneration of allergen-specific effector T cells.

The presence of allergen-specific, afferently acting suppressor cells in orally tolerized animals was confirmed in lymphoid-transfer experiments. In mice and rats oral tolerance is thought to be mediated by suppressor T cells (Ts) [3,7,10,12], presumably of the CD8+ phenotype [18,50]. Still, however, remarkably little is known about the immunoregulatory events that lead to the activation of such suppressor cells and the role of the microenvironment in this process. Reportedly, Ts could be generated in Peyer's patches, via a T suppressor-inducer cell, and subsequently migrate to mesenteric lymph nodes and the spleen [13,51–53]. The finding that allergen entry through oronasal and/or pharyngeal mucosae also induces tolerance argues, however, strongly against a *crucial* role for Peyer's patches [54]. Indeed, it was found that rats depleted from Peyer's patches by surgery were fully susceptible to the induc-

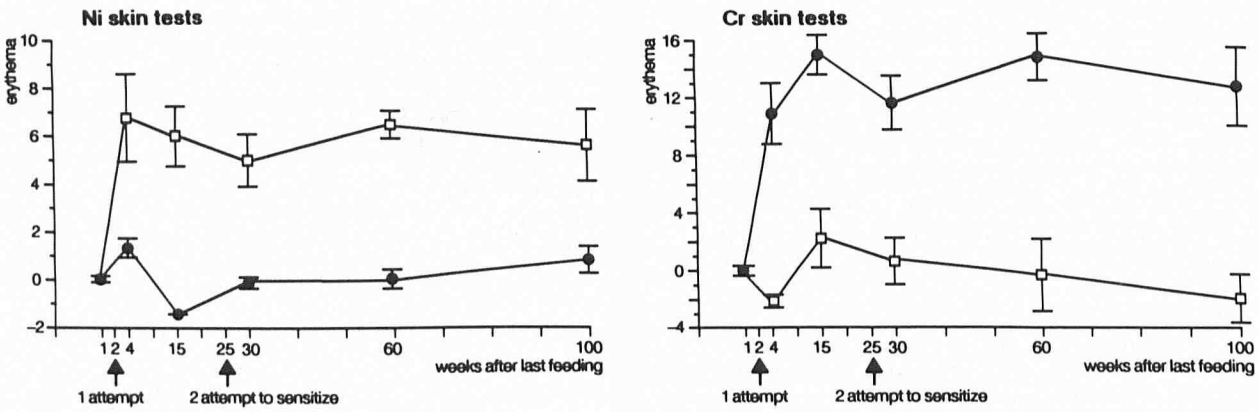

Figure 8. Persistence of immunosuppression; repeated challenges/attempts to sensitize. One group of guinea pigs was fed with 30 mg $NiSO_4$ (●) and another group with 30 mg $K_2Cr_2O_7$ (□) weekly for 6 weeks. A first attempt to sensitize was made with both nickel and chromium at 2 weeks after the feeding period. Half a year later a second attempt to sensitize was made with nickel in the nickel-fed group and with chromium in the chromium-fed group. Intradermal challenges were performed repeatedly to monitor the development of nickel *(left)* and chromium *(right)* hypersensitivity. Data are expressed as mean product score of diameter and intensity of erythema (±SEM) of six guinea pigs per group corrected for the reactivity in control groups immunized with FCA only, at 48 h after challenge.

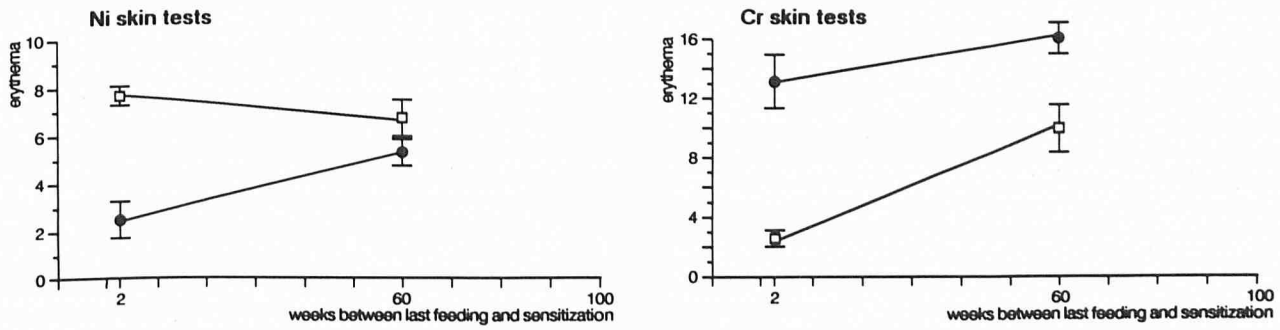

Figure 9. Persistence of immunosuppression; delayed attempt to sensitize. Two groups of guinea pigs were fed with 30 mg $NiSO_4$ (●) and two other groups with 30 mg $K_2Cr_2O_7$ (□) weekly for 6 weeks. Two weeks after the feeding period sensitization to both metals was attempted in one of the nickel-fed and in one of the chromium-fed groups. In the remaining groups the sensitizing attempt was performed 60 weeks after the feeding period. The groups were challenged intradermally 2 weeks after the sensitizing attempt to reveal eventual development of nickel *(left)* and chromium *(right)*. Data are expressed as mean product score of diameter and intensity of erythema (±SEM) of seven to ten guinea pigs per group corrected for the reactivity in control groups immunized with FCA only, at 48 h after challenge.

tion of oral tolerance [55]. Evidence is accumulating now that oral tolerance induction may result from several factors acting in concert, notably a predominance of CD8+ T cells within the mucosal epithelia [56,57], distinct ways of antigen processing [58,59], and patterns of local cytokine release [60]. Here we confirmed that Peyer's patches are not necessarily involved in oral tolerance induction, because a brief and timely application of nickel or chromium salts to the oral mucosa induced strong immune tolerance.

Interestingly, when studying ovalbumin-specific cell-mediated immunity and T-dependent IgE production in rats, Holt and colleagues also noted that the first part of the alimentary tract plays a major role in oral tolerance induction. It was shown that after sublingual administration up to ten-fold lower dosages of antigen were required for tolerance induction as compared to intragastric administration [61]. When comparing oral and intragastric administration in the present study, nickel or chromium salts were brought into contact with the mucosa of the oral cavity only for a short period of time, and care was taken that no allergen was swallowed. Our results support, therefore, the view that the oronasal and/or pharyngeal mucosae are particular targets for the preferential induction of immunologic tolerance [61]. Notably, this may also have contributed to the remarkable tolerogenic effect of orthodontic treatment observed in our recent epidemiologic study [42].

In conclusion, it is shown that specific immune tolerance can be induced by timely oral contacts with metal allergens, and that full unresponsiveness is not broken, but rather strengthened, by repeated skin contacts with the allergens. Without any further oral contacts full tolerance could be maintained for at least 2 years. Because very low doses of allergens, if administered for longer time periods, can induce tolerance, clinical applications of these principles in high risk groups would seem feasible. With the frequencies of nickel allergy still on the rise, experiments are currently designed to evaluate, in a prospective way, whether consumption of food items with high nickel contents at an early age may reduce the subsequent development of subsequent nickel allergies. Further immunotherapies in clinical allergies and autoimmune diseases should also take advantage of the particular tolerogenicity of sublingual antigen (allergen, peptide) applications.

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