

Fig. 2. Immunological response of the Ni-Ti alloy sensitized GATA-3 Tg mice to a Ni challenge. **a** Serum IgE, IgG1 and IgG2a levels in pre-immunized GATA-3 Tg and WT mice and 16 h after a Ni challenge in sensitized GATA-3 Tg and WT mice. No significant difference in the serum level of IgG1 or IgG2a was noted between the Ni-Ti alloy-sensitized/Ni-challenged GATA-3 Tg and WT mice. **b** Cytokine levels in the skin tissue supernatants in the Ni-challenged GATA-3 Tg and WT mice. Data are the means \pm SD in 5 mice per group and are representative of 3 independent experiments. $p < 0.05$ vs. Ni-challenged WT mice.

the edema was not intense in the WT mice (fig. 3b). A very severe inflammatory response with intense infiltration by mononuclear cells, neutrophils and mast cells was observed in the GATA-3 Tg mice, whereas only a mild inflammatory response with little infiltration by mononuclear cells and neutrophils was observed in the challenged

skin of the WT mice. When the number of mast cells infiltrating the dermis was evaluated by staining the tissue specimens with toluidine blue, the number in the Ni-Ti alloy sensitized/Ni-challenged GATA-3 Tg mice (24 ± 2.7) was 3-fold higher than that in the control WT mice (7 ± 5.0) (fig. 3c).

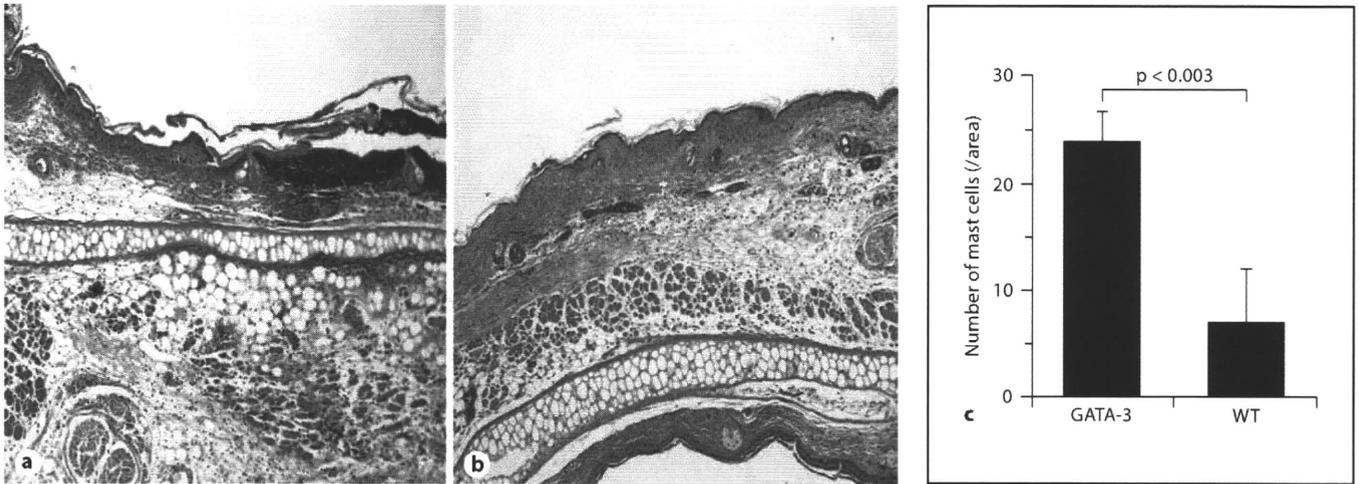


Fig. 3. Histological examination. Ear skin specimens were excised and fixed in 10% formalin, then processed and stained with hematoxylin and eosin. **a** The dermis of the challenged skin in the GATA-3 Tg mice showed a very severe inflammatory response, with infiltration by mononuclear cells, neutrophils and mast cells. **b** WT mouse. **c** The number of mast cells infiltrating the dermis was evaluated by staining the tissue specimens with toluidine

blue. The sections were examined at a magnification of $\times 400$. At least 10 fields per section were examined for each lobe. The cells were counted and expressed as the number of cells per square millimeter area. The bars represent the number of mast cells. Data are the means \pm SD of the counts in groups of 10 mice per group. $p < 0.003$ vs. Ni-challenged WT mice. The photographs of the ears were taken 2 days after the antigen challenge.

Effect of GATA-3 Tg-Purified CD4+ T Cells in Transferring the Ni-Induced Contact Hypersensitivity Reaction

Rag-2^{-/-} mice were transfused with CD4+ T cells from the Ni-Ti alloy-sensitized GATA-3 Tg mice or WT mice. Rag-2^{-/-} mice transfused with CD4+ splenic cells isolated from the Ni-Ti alloy sensitized GATA-3 Tg mice showed significantly greater ear swelling responses than the WT mice at various time-points after the challenge. The peak ear swelling response was observed at 48–72 h after the Ni challenge in both the GATA-3 Tg mice and WT mice (fig. 4a). These results suggest that the CD4+ splenic T cells of the Ni-Ti alloy sensitized GATA-3 Tg mice regulated the ear swelling response more significantly than the CD4+ splenic T cells from the Ni-Ti alloy sensitized WT mice. After the CD4+ splenic T cells derived from non-Ni-Ti alloy sensitized GATA-3 or WT mice were transfused into Rag-2^{-/-} mice, the ear swelling response following challenge with Ni solution followed a similar time-course to that in the Rag-2^{-/-} mice not infused with the cells (data not shown).

Indirect Immunohistochemical Staining for CD4+

Immunohistochemical staining showed severe infiltration by CD4+ cells in the ear skin lesions of the Rag-2^{-/-} mice infused with the CD4+ splenic T cells isolated

from the Ni-Ti alloy sensitized GATA-3 Tg mice, but not in the Rag-2^{-/-} mice infused with CD4+ T cells isolated from Ni-Ti alloy sensitized WT mice (fig. 4b).

Reduced Ear Swelling Response to Ni Challenge and Reduced Serum IgE Level in GATA-3 Tg Mice Sensitized with the Ni-Ti (#500) Test Piece Subjected to Oxygen Diffusion Processing

The ear swelling response to the implantation of a Ni-Ti (#500) test piece subjected to oxygen diffusion processing was about 1/30th to 1/20th of that elicited in response to the implantation of an untreated Ni-Ti test piece (fig. 5). The IgE antibody in the serum of the treated Ni-Ti (#500) test piece-implanted GATA-3 Tg mice was about 60% lower than in the GATA-3 Tg mice implanted with the untreated test piece (table 1).

Degree of Ni Ion Elution from the Oxygen Diffusion-Processed Ni-Ti Alloy

The degree of Ni ion elution from the untreated Ni-Ti test piece was 2–2.5 times greater than that from the Ni-Ti (#500) test piece subjected to oxygen diffusion processing (fig. 6). This finding suggested a correlation between the solubility of the Ni ion and the degree of the ear swelling response.

Discussion

Contact hypersensitivity reactions are mainly thought to be associated with the activation of Th1 cells. However, since evidence of the involvement of Th2 cells and Th2 cytokines has also been reported in the development of contact hypersensitivity [23, 24], in this study, we investigated whether Th2-type immune responses might also be involved in the pathogenesis of ACD. We recently established a GATA-3 Tg mouse model to study the role of Th2-type immune responses in the absence of the suppression of Th1-type immune responses [19]. In this study, we used the GATA-3 Tg mouse model to study the role of the Th2 cytokines IL-4, IL-5, and IL-13 in the development of ACD. At 48–72 h after antigen challenge, the ear swelling response in the GATA-3 Tg mice was significantly greater than that in the WT mice, and the maximal ear swelling response persisted until 320 h after the challenge. The kinetics of the ear swelling responses in the GATA-3 and WT mice were similar, but the magnitude of the response was about three-fold greater in the GATA-3 Tg mice. We also assessed, in another study, the development of 2,4,6-trinitrochlorobenzene-induced contact dermatitis in the GATA-3 Tg mice. Our results also revealed the involvement of Th2-type immune reactions in 2,4,6-trinitrochlorobenzene-induced contact dermatitis, consistent with the results of the present study. The ear swelling reaction in the aforementioned study, however, ended at 48 h after stimulation with the antigen, unlike in the present study. In the experiment involving CD4⁺ cell implantation into Rag-2^{-/-} mice, the response ended at 48 h after the challenge, consistent with other results of the present study. The reasons for the differences are not clear, but the following two explanations should be considered: (1) the number of the cells implanted was not adequate to induce Th1-type cytokine production, or (2) the antigen test substance was present *in vivo* even during the determination of the responses in the present experiment. Taking into consideration the adequate production of IFN- γ in the ear tissue at 48 h after stimulation with the antigen, however, the persistent *in vivo* existence of the antigen used for sensitization was considered to be the major reason for the difference in the duration of the response. Further detailed studies are, however, needed to solve this question. Clarifying this difference is also important from a clinical standpoint and for understanding the possibility of differences in the duration of responses among patients with the same disease, namely, metallic allergy. Our results for the kinetics of ear swell-

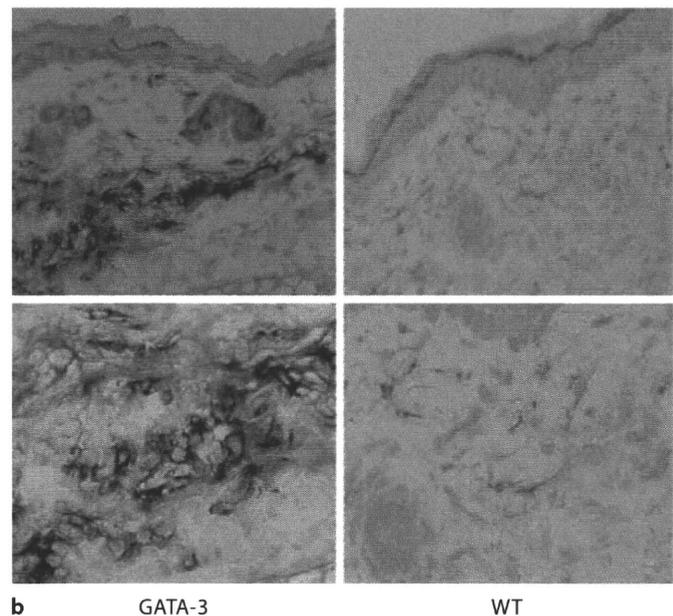
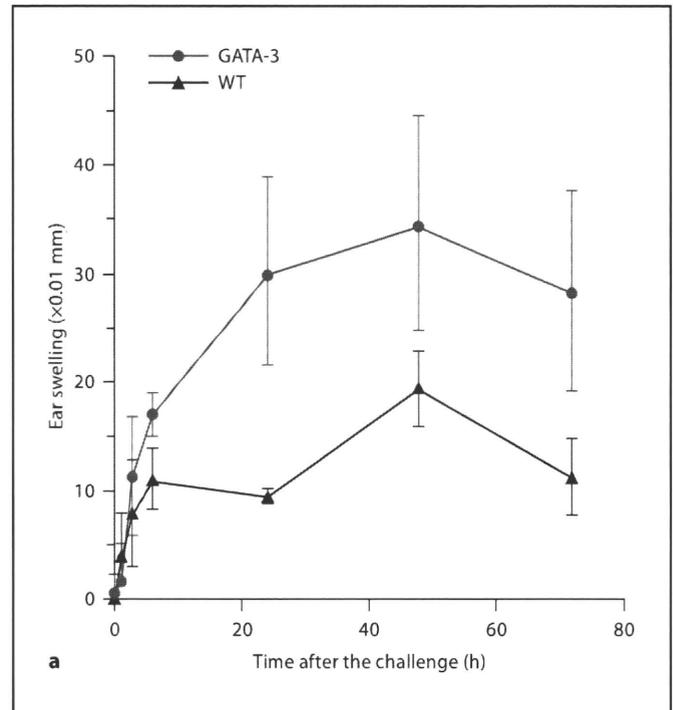


Fig. 4. Immune response of Rag-2^{-/-} mice implanted with CD4⁺ cells isolated from the spleen of the Ni-Ti sensitized GATA-3 Tg mice. **a** The increases in the ear skin thickness of the Rag-2^{-/-} mice implanted with CD4⁺ splenic cells isolated from the GATA-3 Tg or WT mice were measured at 0.5, 1, 3, 24, 48 and 72 h after Ni challenge. Data are the means \pm SD of 5 animals per group and are representative of three independent experiments. $p < 0.05$ vs. WT mice. **b** Sections of Ni-challenged ears from the Rag-2^{-/-} Ni-Ti alloy sensitized GATA-3 Tg and WT mice and nontreated Rag-2^{-/-} mice were analyzed by staining for anti-mouse CD4. The sections were observed at magnifications of $\times 200$ and $\times 400$.

Fig. 5. Immune response (ear swelling) in GATA-3 Tg mice transplanted with the Ni-Ti (#500) test piece. The ear skin thickness of the GATA-3 Tg mice implanted with the Ni-Ti (#500) test piece subjected to oxygen diffusion processing was measured at the time-points indicated after the subcutaneous injection of 10 μ l of a solution of Ni (1,000 ppm) into the left ear and 10 μ l of saline into the right ear. The differences in the thickness between the right and left ears after the challenge are shown. Data are the means \pm SD of 5 animals per group and are representative of 3 independent experiments. $p < 0.05$ vs. WT mice implanted with nontreated Ni-Ti alloy. ● = GATA-3 Tg mice implanted with non-treated Ni-Ti alloy; □ = WT mice implanted with nontreated Ni-Ti alloy; ■ = GATA-3 Tg mice implanted with the Ni-Ti alloy subjected to oxygen diffusion.

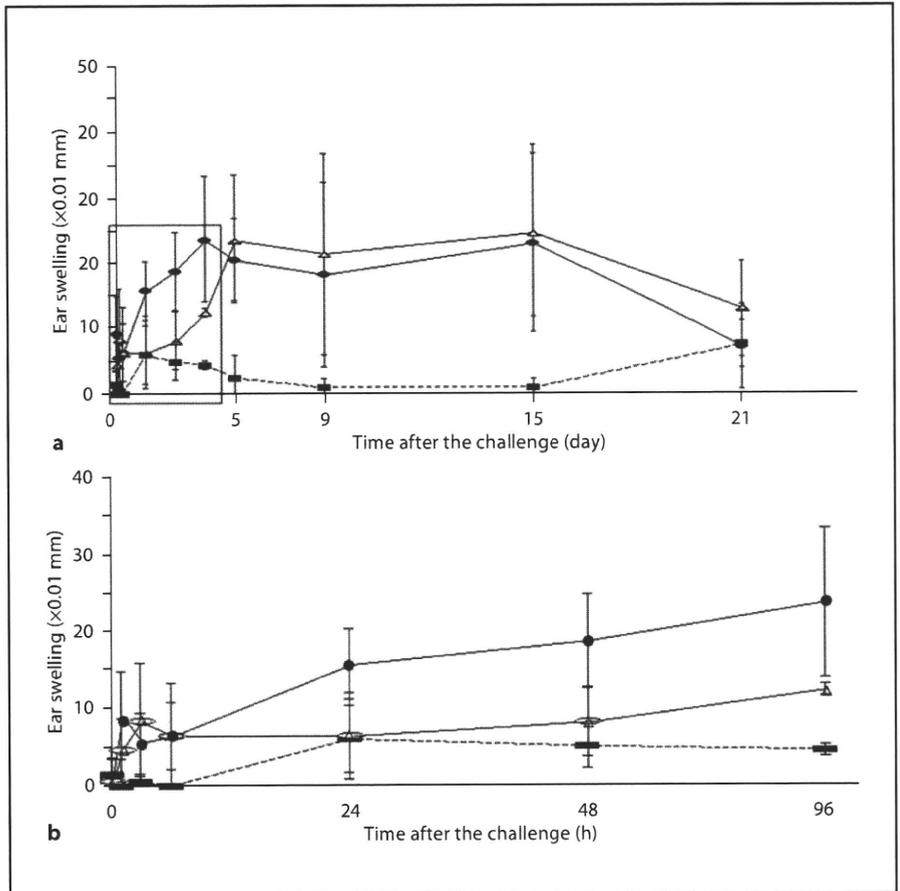


Table 1. Serum IgE levels in the GATA-3 Tg mice implanted with the Ni-Ti (#500) test piece subjected to oxygen diffusion processing

Ni-Ti (treatment)	Total IgE, ng/ml	Reduction, %
-	762 \pm 343.7	
+ (none)	1,489.8 \pm 843.1	0
+ (#500)	605.3 \pm 592.0	59.3

Data are means \pm SD for groups of 5 mice and are representative of 3 independent experiments.
 $p < 0.05$ vs. GATA-3 mice not implanted with the test piece.

ing in the GATA-3 Tg mice after antigen challenge reflected a Th2-type immune response (IL-4) to the Ni-Ti alloy sensitization as well as a Th1-type response (IFN- γ) suppressed by the Th2-type response. These findings suggest that while both Th1- and Th2-mediated im-

mune responses were key factors in the development of the ACD in the GATA-3 Tg mice, the Th2 cytokines (for example IL-4) were particularly important for the induction of ACD in this model. The Th1-type immune response is also thought to be ultimately important for the development of dermatitis induced by Ni. In every one of our experimental systems, the amount of IL-4 produced in vivo was only 1/40 to 1/20 of the amount of IFN- γ produced. Since the amount of IL-4 produced was markedly lower than that of IFN- γ , the effects of the former could also be expected to be weaker. However, the effects of the cytokines became clear only after a comparison of not only the amount of production, but also the functions of IL-4 and IFN- γ . In the present study, the level of IL-4 produced in the GATA-3 Tg mice was approximately twice that produced in the WT mice at 48 h after stimulation with Ni solution, and the level of IFN- γ produced in the same mice was half of that in the WT mice. However, from 96 h onward after the stimulation, the tissue production of IFN- γ was sustained at 20,000–

25,000 pg/g tissue until the reaction ended, while the production of IL-4 decreased markedly in both the GATA-3 Tg mice and WT mice.

As shown in figure 4a, the Rag-2^{-/-} mice infused with the CD4⁺ splenic cells isolated from the GATA-3 Tg mice showed marked ear swelling. The following mechanisms underlying this result have been proposed: (1) the induction of numerous memory cells to the spleens of the GATA-3 Tg mice, or (2) a higher activity level of the CD4⁺ splenic cells in the GATA-3 Tg mice than in the WT mice following Ni challenge, etc. However, the exact mechanisms remain unclear at the present time, and this issue will require further detailed studies in the future. Histopathological examination of the site of inflammation showing infiltration by the CD4⁺ cells and mast cells suggested that a Th2-type immune response was also secondarily involved in the development of the ACD. We confirmed the accumulation of the CD4⁺ splenic cells in the Ni-Ti alloy sensitized GATA-3 Tg mice following the Ni challenge, with an approximately four-fold higher number of these cells in the GATA-3 Tg mice than in the WT mice (data not shown). The results of the histopathological examination of the GATA-3 Tg mice also indicated that the decrease in IFN- γ production promoted fibrosis. These observations suggest that the Th1-type response was suppressed by the increase in IL-4 production as a part of the Th2-type immune response. IL-4 is known to be produced not only by T cells, but also by natural killer T cells, basophilic cells and mast cells. However, the results of an experimental system using the Rag-2^{-/-} mice in this study showed the close involvement of the CD4⁺ splenic cells in the immediate-phase responses (24–48 h) after stimulation with the Ni solution in the Ni-Ti alloy sensitized GATA-3 Tg mice. This finding indicates that the T cells are the main IL-4-producing cells, even though this cytokine might also have been produced by other cells. In another experimental system using the lck distal promoter with the aim of specifically expressing transgenes in the T cells, a marked CD4⁺ cell response was seen in the GATA-3 Tg mice, indicating the major importance of the T cells. In the GATA-3 Tg mice, IL-4 production was significantly promoted by stimulation with the Ni solution, resulting in the suppression of IFN- γ production by the action of the former on macrophages. The production of IL-4 itself is considered to decrease with time, leading to the elimination of its suppressive effect on IFN- γ production. As a result, the levels might reach almost the same levels as those seen in WT mice over time, and IFN- γ and IL-4 might show similar time-course changes. The decrease

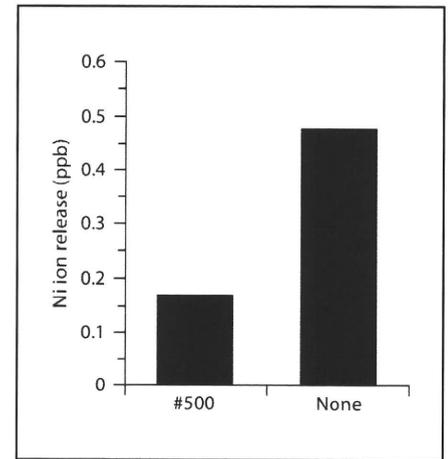


Fig. 6. Examination of Ni ion elution after oxygen diffusion processing. The test piece was subjected to oxygen diffusion processing and soaked in 3% NaCl solution maintained at 37°C for 28 days. The density of the eluted Ni ions in the solution was then measured using atomic absorption photometry.

is regarded as a specific reaction occurring in part as a result of the stimulation with the Ni solution, since no inflammatory cell infiltration was recognized in a histopathological examination of the tissues after Ni-Ti alloy implantation performed after a challenge with the Ni solution.

An investigation of the cytokines in the tissues showed that the immediate-phase responses in the Ni-Ti alloy-implanted GATA-3 Tg mice were in fact Th2-type immune reactions. We think that with time, the Th1-type immune reactions also contributed to the production of a major swelling reaction, because both GATA-3 Tg mice and WT mice started showing similar swelling reactions by 96 h after stimulation with Ni. Pathological examination of the tissues after the reactions subsided revealed marked fibrosis of the ear tissue in the GATA-3 Tg mice, which showed significant IL-4 production during the immediate-phase responses (data not shown). In the WT mice, which showed a significantly larger production of IFN- γ than of IL-4, no fibrotic changes were observed, and the changes were restored to the findings observed in the normal tissues. These data indicate that the IL-4 produced during the immediate-phase responses induces dermal fibrosis and that its production is suppressed by IFN- γ . The extent of the participation of the Th2-type immune reactions in the development of allergy has been suggested to influence the prognosis of Ni-induced allergy. Under this circumstance, the use

of Th2-type immune reactions as an indicator might influence the treatment policies used for subjects with metal allergies. Some reports have shown that IL-4 is important for the occurrence of fibrosis of the liver during infection with parasites and bleomycin-induced pneumonia [25, 26]. A previous report has also shown that IL-13 is more important than IL-4 [27]; however, no difference in IL-13 production was observed between the GATA-3 Tg mice and the WT mice in the present study, suggesting that IL-4 production plays a major role in the development of fibrosis in the present experimental system.

Th2 cell differentiation is mediated by signal transducer and activator of transcription (STAT) 6 activation induced by the engagement of the IL-4 with its receptor [28–30]. Asherson et al. [31] reported that IL-4 plays an important role in the pathogenesis of contact hypersensitivity. They speculated that the IL-4 secreted by mast cells or B220⁺ cells binds to γ/δ T cells and induces these cells to secrete more IL-4 or other cytokines that increase the expression of adhesion molecules on the endothelial cells, which in turn are believed to play a critical role in the induction of contact hypersensitivity. In contrast, Berg et al. [32] demonstrated the induction of contact hypersensitivity to 4-ethoxyl methylene-2-phenyl-2-oxazolin-5-one (Oxa) in IL-4-deficient mice. Traidl et al. [33] found that the complete loss of endogenous IL-4 expression in BALB/C mice was associated with an impaired manifestation of contact hypersensitivity to 2,4-dinitrochlorobenzene, but not to Oxa. Yokozeki et al. [23] recently established a strain of STAT6-deficient mice and demonstrated that STAT6 plays a central role in IL-4- and IL-13-mediated biological responses. They found that the contact hypersensitivity responses to 2,4-dinitrochlorobenzene and Oxa were significantly attenuated in STAT6-deficient mice and that the peak response was also delayed. The STAT6 signal induces the expression of the transcription factors GATA-3 and c-Maf [34], which are reportedly selectively expressed in a Th2-specific fashion [14, 15, 30]. In a recent study of STAT6-deficient cells, it was shown that although IL-4 and STAT6 signaling might initially direct the development of the Th2-type response, GATA-3 and c-Maf are also capable of inducing a stable Th2 commitment, independent of STAT6 [35]. In vitro differentiation into Th2 cells induces the remodeling of the IL-4/IL-5/IL-13 locus. GATA-3 plays an essential role in the earliest stages of T-cell development [36, 37] and has also been recognized as a Th2 differentiation factor [16]. GATA-3 has been detected in naive CD4⁺ T cells, and its expression

level continues to increase substantially during Th2 differentiation [14, 15]. GATA-3 expression is reportedly indispensable for Th2 development and is down-regulated in response to IL-12-mediated STAT4 activation [14, 38]. GATA-3 strongly transactivates the IL-5 and IL-13 promoters but appears to have only limited effects on IL-4 gene transcription [14, 17, 38]. The results of experiments conducted to date suggest the possibility that the GATA-3 gene expression level varies with the amount of the antigen, the method of immunity induction, and the duration of stimulation, although it is not clear whether the gene controls the level of IL-4 production. The results of the present experiment are consistent with the aforementioned suggestions.

In the present study, we demonstrated that Th2 cells and Th2-type cytokines are involved in the pathogenesis of the Ni-induced ACD response. In the GATA-3 Tg mice, the late-phase ear swelling response to a Ni challenge was significantly more pronounced than the immediate-phase ear swelling response. At the site of the challenge in the GATA-3 Tg mice, the local production of IL-4 was increased and INF- γ production was suppressed. These findings suggest the involvement of an IL-4-dominant Th2 response in the development of ACD to Ni. This murine model is characterized by the predominant production of IgE antibody and no IgG1 or IgG2a production, and the induction of the ACD to Ni was markedly suppressed when a Ni-Ti alloy subjected to oxygen diffusion processing was implanted. Whether the antibody titers were Ni-specific remains uncertain. However, the IgE antibody titers did not increase following the transplantation of the Ni-Ti alloy test piece in the GATA-3 Tg mice. In recent years, many reports have shown that allergens not only induce antigen-specific IgE antibody production, but also act like a Th2-type adjuvant, inducing non-antigen-specific immune reactions [39, 40]. Under this circumstance, Ni might represent a nonspecific response to Th2-type adjuvant in Ni-induced ACD, although the mechanism remains unclear. However, we believe that Ni-specific antibody was involved. These results suggest that Th2 cells and Th2 cytokines are important for the ear swelling response during the late phase of the development of ACD. The GATA-3 Tg murine model might be useful for increasing our understanding of the physiological significance of the Th2 cells and Th2 cytokines in ACD induced by Ni. When a Ni-Ti alloy subjected to oxygen diffusion processing was used, the ear swelling response was suppressed, indicating that the ear swelling response is increased independent of the concentration of

the Ni ions transplanted into the living body and that the suppression of the response is an indicator of the decrease in the allergy-inducing activity of the contact sensitizer. These findings suggest that the use of the GATA-3 Tg mice established by us may be of great use in the development of biomaterials with low allergy-inducing activity.

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