Brief Definitive Report

Induction of Hapten-specific Tolerance by Interleukin 10 In Vivo

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Summary

Interleukin 10 (IL-10) is released during the induction phase of contact sensitivity and was shown in prior functional studies to convert epidermal Langerhans cells (LC) from potent inducers of primary immune responses to specifically tolerizing cells in vitro. To investigate whether IL-10 also subserves the function of a tolerizing agent in vivo ears of BALB/c or C3H mice were injected intradermally with 1–2 μg of recombinant mouse (rm)IL-10 8 h before epicutaneous application of 3% trinitrochlorobenzene (TNCB; a contact allergen). As a control, mice were injected with phosphate-buffered saline or IL-10 plus neutralizing amounts of anti-IL-10 mAb. 5 d later, mice were challenged with 1% TNCB on contralateral ears and ear swelling response was measured 24 h later. Whereas control-treated mice showed a normal ear swelling response to epicutaneous challenge (Δ mm^{-2} = 25 ± 5), ear swelling response of IL-10–treated animals was significantly inhibited (Δ mm^{-2} = 3 ± 2). Coinjection of IL-10–specific mAb together with rmIL-10 completely abrogated this effect. To differentiate between a state of nonresponsiveness and induction of tolerance by IL-10, mice initially treated with IL-10 and TNCB were resensitized with 3% TNCB in the absence of any treatment after 14 d of rest (group 1). Again mice were challenged 5 d later and ear swelling responses were tested. Whereas control mice treated with allergen alone (group 2) showed a good swelling response (Δ mm^{-2} = 28 ± 6), IL-10–treated mice (group 1) showed a minimal response towards application of allergen (Δ mm^{-2} = 4 ± 2). To show that anergy induction by IL-10 was antigen-specific, mice initially treated with IL-10 plus TNCB were exposed to 0.5% dinitrofluorobenzene (DNFB) 14 d later (group 1). After challenge with 0.1% DNFB, IL-10–treated mice showed an ear swelling response (Δ mm^{-2} = 13 ± 3; group 1) similar to that of control mice only sensitized with DNFB (Δ mm^{-2} = 14 ± 3; group 3). In an attempt to show the induction of antigen-specific tolerance in these mice in vitro, regional lymph nodes of mice initially treated with TNCB plus IL-10 (group 1) and control-treated mice (groups 2 and 3) were prepared and cultured in the presence of TNBS, dinitrobenzene sulfonate (DNBS), or medium to measure antigen-specific proliferation. Lymph node cells from animals sequentially treated with IL-10 plus TNCB and afterwards DNFB in the absence of IL-10 (group 1) showed a low, but significant proliferation (~15,000 cpm) to DNBS, but only background proliferation (~3,000 cpm) to TNBS, or medium. In contrast, lymph node cells from animals treated with TNCB or DNFB in the absence of IL-10 (groups 2 and 3) proliferated to the sensitizing agent, but not control allergens. To elucidate the mechanism of action of IL-10, the epidermal cytokine pattern was analyzed on the mRNA level after injection of IL-10 or controls and application of allergen. Injection of IL-10 (but not controls) significantly impeded the induction of proinflammatory cytokines IL-1β, tumor necrosis factor α and IL-1α. In aggregate our data indicate that in vivo application of IL-10 before allergen treatment induces antigen-specific tolerance in mice and that IL-10 might act via inhibition of proinflammatory cytokines.

IL-10 was originally identified as a product of Th2 cell clones inhibiting the proliferation of Th1 cell clones via downregulation of IFN-γ and IL-2 production (1, 2). These effects are dependent on the presence of viable APCs and their extent varies depending on the kind of APCs used (3). Initial studies performed with macrophages, B cells, or blood dendritic cells showed a significant inhibitory influence of IL-10 on the APC functions of macrophages only (4–6). In this system the inhibitory influence of IL-10 on the proliferation of Th1 cell clones was thought to be mediated by downregu-

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lation of MHC class II molecules. In addition, De Waal Malefyt et al. (14) discussed an inhibitory influence on costimulatory molecules. They demonstrated that the inhibition of MHC class II molecules on human monocytes by IL-10 could be reversed by IL-4. The inhibitory effect on the proliferation of T cells on the other hand remained unaffected (4). These findings were supported by studies of Ding et al. (5) who reported a profound effect of IL-10 on macrophage accessory functions in assay systems independent of MHC class II expression. This group recently related the IL-10 effect on macrophages to an inhibition of B7 expression (7).

Recently it was shown that IL-10 is also a product of activated keratinocytes and is released during the induction phase of contact sensitivity (8). Further studies could define the effects of IL-10 on dendritic Langerhans cells (LC) as the APC in the epidermis (9). It was demonstrated that IL-10 inhibited the induction of proliferation of Th1 cell clones by freshly prepared LC. This effect was independent of Ag-processing and shown to be mediated via inhibition of a costimulatory signal on LC (but not B7 or intercellular adhesion molecule 1 (ICAM-1) [9]). In fact LC pretreated with IL-10 were converted from specifically sensitizing to specifically tolerizing APC in vitro. The present study was designed to demonstrate tolerizing effects of IL-10 in vivo.

Materials and Methods

Animals. BALB/c and C3H/HeN mice were purchased from the Zentralinstitut für Versuchstierhaltung (Hannover, Germany). They were used at 8–12 wk of age.

Chemical Treatment. Trinitrochlorobenzene (TNCB) was purchased from Polysciences, Inc. (Warrington, PA), dinitrofluorobenzene (DNFB) was purchased from Sigma Chemical Co. (St. Louis, MO). Trinitrobenzenesulfonate (TNBS) and dinitrobenzenesulfonate (DNBS) were purchased from Eastman Kodak Co. (Rochester, NY). For in vivo induction of anergy mice were anesthetized and 1–2 μg of recombinant mouse (rm)IL-10 (kindly provided by M. Howard and S. Menon, DNAX Research Institute, Palo Alto, CA) or IL-10 together with 30 μg of anti-IL-10 mAb (PharMingen, San Diego, CA), or PBS were injected intradermally with a 30-g needle into the ears of naive mice. 8 h later, 5 μl of 3% TNCB (dissolved in acetone) were applied over the injection sites taking special care not to spread the allergen. 5 d later ears were challenged with 1% TNCB and ear swelling responses were measured 24 h later with an engineer's micrometer (Oditest®). IL-1–treated mice were then given a rest of 14 d and then resensitized anew in the absence of any treatment as described before (10). After a final rest of 14 d the same animals were exposed to 0.5% DNFB on 2 d consecutively and challenged with 0.1% DNFB 4 d later. Ear swelling responses were measured 24 h later. Control groups were mice sensitized and challenged with TNCB or DNFB in the absence of any treatment, or just challenged animals. All experiments were performed at least in triplicate with six mice per group.

Lymph Node Proliferation Assays. Draining regional lymph nodes from animals treated with TNCB and IL-10 as described above or control-treated animals were removed immediately after determination of swelling responses, mashed, and either pulsed with TNBS, or DNBS (water soluble analogues of DNCB and DNFB), or medium as described (11). Cells were then aliquoted at 2×10^5 cells/well in 96-well plates (Becton Dickinson & Co., Oxnard, UK) and proliferation was determined by addition of 1 μCi [^{3}H]thymidine for the last 12–16 h of the 96 h incubation.

PCR and Liquid Hybridization. EC suspensions were prepared as described previously (12) and total epidermal RNA was extracted by RNAzol B (Paesel und Lorei, Frankfurt, Germany) following the instructions of the manufacturer. PCR amplification was performed according to Saiki et al. (13) with cycling conditions chosen at 1 min at 95°C, 1.5 min at 55°C, and 2 min at 72°C and 25 cycles using the Perkin Elmer RNA-PCR kit (Perkin-Elmer, Überlingen, Germany). Primers were designed according to published sequences and span one intron (10). Primer and RNA concentrations, as well as PCR cycles were titrated to establish standard curves to document linearity and to permit quantitative analysis of signal strength. 5 μl of amplified PCR product were hybridized to an excess of ^{32}P-endlabeled probe as described (10). All probes consist of internal sequences of our specific PCR products. After hybridization, samples were loaded on 4% PAGE gels, dried, and autoradiographed. Omission of reverse transcriptase controlled for DNA contamination.

Statistical Analysis. A Student's *t* test was performed to document interpretability of ear swelling responses.

Results and Discussion

Inhibition of Primary Sensitization by IL-10. To demonstrate inhibition of sensitization in vivo, mice were anesthetized and injected with 1–2 μg rmIL-10 in 50 μl sterile PBS or controls. 8 h later ears were treated with 5 μl 3% TNCB above the injection site, taking special care not to spread the allergen. 5 d later 1% TNCB was applied to contralateral ears and ear swelling was determined 24 h later with an engineer's micrometer (Fig. 1). Whereas control-treated mice (injected with PBS or IL-10 plus saturating amounts of neutralizing anti-IL-10 mAb) showed a good swelling (Δ mm^{-2} = 25 ± 5) response towards allergen treatment, animals in-

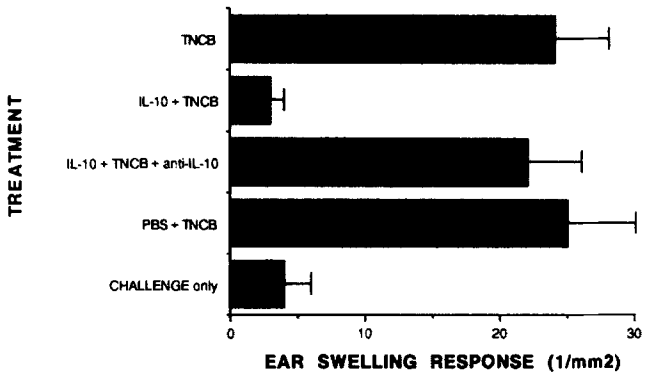

Figure 1. Inhibition of primary sensitization in mice by IL-10. Mice were injected intradermally into the ears with 2 μg rmIL-10 (IL-10 + TNCB), PBS (PBS + TNCB), or 2 μg IL-10 plus 20 μg of anti-IL-10 mAb (IL-10 + TNCB + anti-IL-10) and painted with 3% TNCB above the injection site 8 h later, or were only treated with 3% TNCB (TNCB) without prior injection. 5 d later mice were challenged with 1% TNCB applied to contralateral ears and ear swelling was measured 24 h later with an engineer's micrometer. Mice only painted with 1% TNCB on day 5 served as control for nonspecific inflammatory edema caused by chemical painting (challenge only). x-axis shows differences in ear thickness before and after challenge (Δ mm^{-2}).

jected with IL-10 were severely impeded in their swelling reaction (Δ mm^{-2} = 3 ± 2, p <0.001; Fig. 1) showing a state of nonresponsiveness. The challenge only group reflects the amount of nonspecific swelling caused by allergen application alone.

Injection of IL-10 8 h before application of allergen seemed to be crucial for the induction of nonresponsiveness as time course experiments demonstrated that injection of IL-10 at the time of TNCB painting or later failed to induce such an effect. Also injection of IL-10 12 h or later before the application of TNCB was without effect (data not shown). Furthermore, comparatively high doses of IL-10 were needed to see an inhibition of ear swelling responses with doses below 0.5 μg being without effect. Injection of 2 μg of IL-10 proved to be most effective in the induction of IL-10–mediated nonresponsiveness.

In addition, analysis of MHC class II expression of LC after IL-10 injection and TNCB treatment on epidermal sheets demonstrated an upregulation of those molecules after hapten application showing no difference to LC on epidermal sheets treated with contact allergen alone.

Induction of Hapten-specific Tolerance by IL-10. To investigate whether hapten-specific tolerance was induced in IL-10 plus allergen–treated mice, these animals were resensitized after a period of 14 d with 3% TNCB in the absence of any treatment and challenged with TNCB as described. Indeed the ear swelling response of IL-10–pretreated mice remained low (Δ mm^{-2} = 2 ± 2). In contrast, mice treated with allergen only (TNCB) responded normally (Δ mm^{-2} = 22 ± 5, p <0.001, Fig. 2).

To exclude the possibility that allergen plus IL-10–pretreated mice were nonresponsive to any given stimulus, animals were resensitized with a different contact allergen (DNFB) 14 d later in the absence of any treatment. TNCB plus IL-10–pretreated mice behaved like normal controls in their swelling response towards DNFB (Δ mm^{-2} = 13 ± 3 vs. Δ mm^{-2} = 14 ± 2, p <0.001) demonstrating the capability

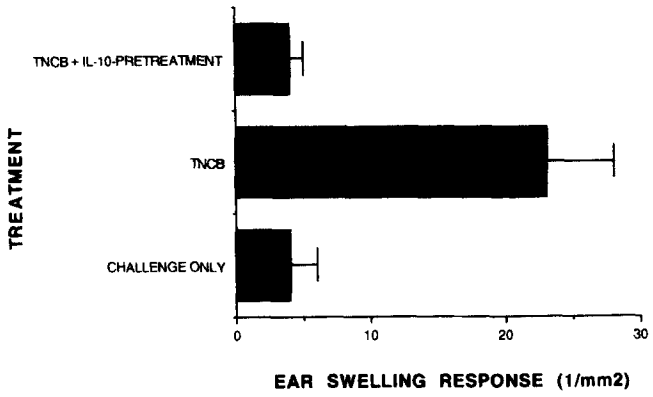

Figure 2. IL-10 induces long-lasting anergy in mice. Mice initially treated with TNCB plus IL-10 (TNCB + IL-10 pretreatment) as described in Fig. 1 as well as control mice (TNCB) were sensitized 14 d later with 3% TNCB. Contralateral ears were painted with 1% TNCB 5 d later and ear swelling responses were measured after an additional 24 h. x-axis shows Δ mm^{-2}.

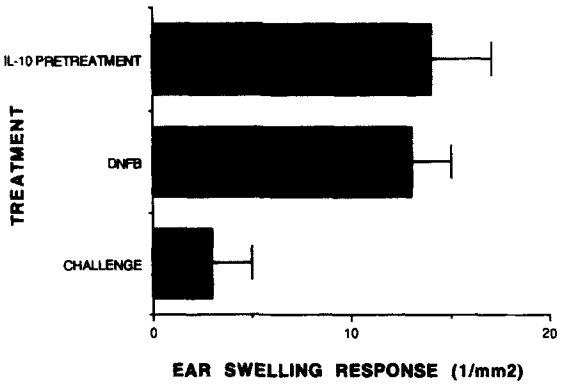

Figure 3. Induction of tolerance by IL-10 is hapten-specific. Mice pretreated with TNCB plus IL-10 as described in Fig. 1 and resensitized 14 d later with TNCB as described in Fig. 2 (IL-10 pretreatment) as well as untreated controls (DNFB) were exposed to 0.5% DNFB on 2 d consecutively. Contralateral ears were treated with 0.1% DNFB 4 d later and ear swelling response measured after an additional 24 h or challenged only. x-axis shows Δ mm^{-2}.

of these animals to launch immunological responses in principal (Fig. 3).

Hapten-specific Lymph Node Proliferation Is Severely Impeded in IL-10–treated Mice. Draining lymph nodes from mice made anergic with TNCB plus IL-10 pretreatment and resensitized sequentially with TNCB and (14 d later) DNFB (tested by ear swelling responses), or lymph node cells from mice treated only with TNCB or DNFB were prepared and cultured in 96-well plates for 4 d in the presence of TNBS or DNBS or medium. Proliferation was detected by incorporation of [^{3}H]thymidine during the last 12–16 h. Lymph node cells from only TNCB- or DNFB-sensitized mice responded well (~14,000 cpm) towards the respective soluble antigen, but did not show cross-reactivity to the other allergen and did not proliferate to medium alone (~3,000 cpm, data not shown). Lymph node cells derived from animals treated with TNCB plus IL-10 and resensitized with TNCB and DNFB proliferated only to DNBS, but not TNBS (Fig. 4) or medium (not shown). Addition of 100 U/ml of rIL-2 to the TNCB plus IL-10–pretreated TNBS group restored the proliferating capacity of these cells excluding cell death. Our data indicate the induction of hapten-specific tolerance in lymph node cells derived from allergen plus IL-10–treated mice.

Inhibition of Epidermal Cytokines by IL-10. As other investigators have demonstrated an inhibitory effect of IL-10 on monocyte-derived cytokines (14), we wondered whether IL-10–mediated induction of tolerance might be mediated by inhibitory effects on epidermal cytokines known to be essential for the induction of primary immune responses in skin (11). Therefore 1–2 μg of rmIL-10 or IL-10 together with anti-IL-10 mAb were injected into the ears of anesthetized mice 8 h before epicutaneous application of allergen as described above. 4 h after application of allergen EC suspensions were prepared and total epidermal RNA was extracted as described in the Materials and Methods section. This pro-

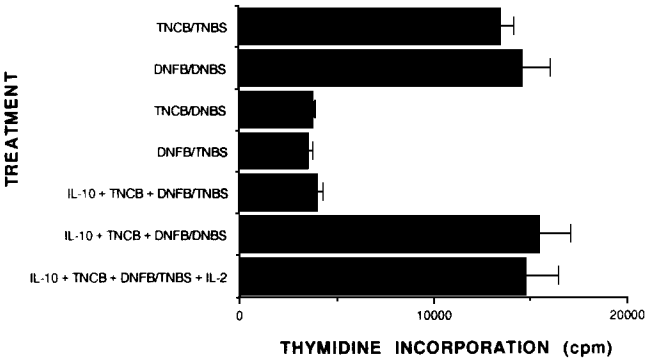

Figure 4. Inhibition of hapten specific lymph node proliferation by IL-10. Draining lymph nodes from animals either treated with TNCB plus IL-10 or control-treated were prepared and incubated with DNBS, TNBS, or TNBS plus 100U rIL-2 for 4 d. 1 μCi [^{3}H]thymidine was added for the last 12–16 h to measure proliferation. Groups are: TNCB-treated animals stimulated with TNBS in vitro (TNCB/TNBS); DNFB-treated animals treated with DNBS in vitro (DNFB/DNBS); TNCB-treated animals stimulated with DNBS in vitro (TNCB/DNBS); DNFB-treated animals stimulated with TNBS in vitro (DNFB/TNBS); animals pretreated with IL-10 and TNCB and resensitized sequentially with TNCB and (14 d later) DNFB and stimulated with TNBS (IL-10 + TNCB + DNFB/TNBS); animals pretreated with IL-10 and TNCB and resensitized sequentially with TNCB and DNFB stimulated with DNBS (IL-10 + TNCB + DNFB/DNBS); and animals pretreated with IL-10 and TNCB and resensitized sequentially with TNCB and DNFB stimulated with TNBS plus 100 U rIL-2 (IL-10 + TNCB + DNFB/IL-2). Background proliferation varied between 3143 ± 324 and 3321 ± 429 cpm.

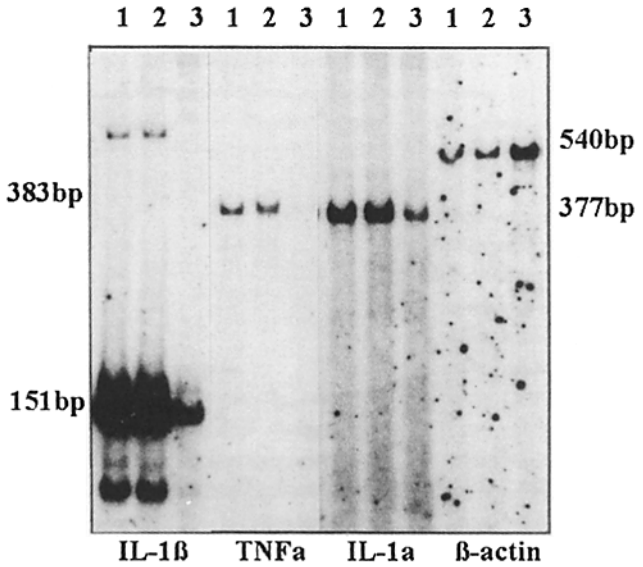

Figure 5. IL-10 downregulates epidermal cytokines. 3% TNCB was applied to the ears of BALB/c mice 8 h after the injection of *1*, nothing; *2*, 2 μg IL-10 plus 30 μg anti-IL-10 mAb, or *3*, 2 μg IL-10 and total epidermal RNA was extracted 4 h later as described. RNA was analyzed by quantitative PCR using primers for IL-1β, IL-1α, TNF-α, or β-actin controls, and subjected to analysis by PAGE, dried, and autoradiographed. Injection of PBS alone did not result in significant signal induction (not shown).

cedure was followed by a quantitative reverse transcriptase PCR using primers for IL-1β, IL-1α, TNF-α, and β-actin controls. Indeed a significant inhibition of mRNA induction could be observed for all cytokines tested after pretreatment with IL-10, whereas β-actin controls remained unaffected (Fig. 5). In this regard the downregulation of IL-1β by IL-10 seems to be of paramount importance. Prior studies have shown that the induction of IL-1β in epidermal LC is essential for inducing primary immune responses in skin (11). IL-1β is the first cytokine (within 15 min of hapten application) to be induced after application of allergen (not irritant or tolerogen). It was also shown to be capable of triggering the whole cascade of cytokines characteristic for the induction phase of contact sensitivity, as well as causing changes in LC morphology, MHC class II expression, and density closely resembling those caused by allergen in vivo. In addition, injection of anti-IL-1β mAb before allergen application prevented sensitization in mice (11), emphasizing the essential role of IL-1β as an important costimulator in primary immune responses in skin. The inhibition of this important molecule by IL-10 might be one of the mechanisms of IL-10 action in skin.

The production of IL-10 by epidermal keratinocytes during the induction phase of contact sensitivity and the rather late upregulation of this cytokine compared with others was shown some time ago (8). Further studies characterized the effect of IL-10 in skin as an inhibition of LC APC function (9). It was demonstrated that IL-10 inhibited the proliferation of Th1 cell clones induced by IL-10–pretreated LC and prevented the upregulation of costimulatory molecules on these cells. Therefore, IL-10 converted LC from potent inducers of primary immune responses to specifically tolerizing cells in vitro (9). In this study we demonstrate that local injection of IL-10 before epicutaneous application of allergen also induces hapten-specific tolerance in vivo. We furthermore demonstrate an effect of IL-10 on the local cytokine milieu of the epidermis during the induction of a primary immune response. In this regard we show that IL-10 inhibits production of mRNA signals for IL-1β, IL-1α, and TNF-α. The observation that the epidermal cytokine pattern of the epidermis is disturbed by IL-10 is of special interest to us as we demonstrated before that the early induction phase of contact sensitivity is characterized by a rather distinct and specific pattern of epidermal cytokines only induced after application of allergen, not tolerogen or irritant application (11). Among all cytokines assessed especially keratinocyte-derived TNF-α (15) and IL-6 (16), as well as LC-derived IL-1β (11, 17) seem to be important. Our own group demonstrated that IL-1β production by epidermal LC is preceding all other cytokines tested. In fact, IL-1β alone is sufficient to induce the whole cascade of allergen-specific cytokines and a monoclonal anti-IL-1β antibody injected into the skin before application of allergen completely prevented epicutaneous sensitization (11). This demonstrates that IL-1β production by LC is indeed essential for the induction of primary immune responses in skin (11). Therefore an inhibition of LC-derived IL-1β by IL-10 might help explain the mechanism of toler-

ance induction in this system. It might be that local production of IL-1β and the following release of proinflammatory cytokines by keratinocytes is needed for the upregulation of certain costimulatory molecules on LC or that these cytokines might serve as costimulators themselves as has been described for IL-1 in macrophage systems (18). This is of special importance as Schuler and Steinman (19) have demonstrated before that resident LC in the epidermis are rather immature with regard to their capability to induce T cell proliferation as compared with dendritic cells derived from lymph node, blood, or spleen (19–21). This is at least partially due to a lack of costimulatory signals on resident LC that need to acquire these molecules during their migration to regional lymph nodes. As absence of costimulation in the presence of antigen-presentation leads to T cell anergy (22), LC leaving the skin under the influence of IL-10 and incapable of upregulating those costimulatory molecules might serve as tolerizing APC, thereby limiting the amount of specifically sensitized T cells. This might serve as a counterregulatory mechanism in primary immune responses in skin to minimize tissue damage.

This theory is supported by data generated by Ding et al. (5) who demonstrated in an MHC class II–independent proliferation assay that IL-10 inhibited a costimulatory molecule on macrophages. In further studies the costimulator was shown to be B7 (7). Although B7 expression does not seem to be affected on LC, our own data clearly demonstrate that some other costimulatory molecule is target of IL-10 action on LC (9).

Recently Rivas and Ullrich (23) showed that intraperitoneal injection of high amounts of supernatant derived from UV-irradiated KC suppressed delayed-type hypersensitivity (DTH) responses in mice as assessed by footpad swelling. The active compound in the supernatants was shown to be IL-10 as an IL-10–specific mAb completely reversed the effect. Our own attempts to induce suppression of contact allergy induction by intraperitoneal injection of IL-10 were rather unsuccessful. Possible explanations for this might be that the amounts of IL-10 reaching the application site of the allergen were insufficient. Although Rivas and Ullrich (23) did not exactly quantify the amount of IL-10 that was injected in their study, Powrie et al. (24), in a very recent report, needed doses in between 20 and 50 μg of purified IL-10 injected i.p. to observe an effect on DTH reactions. This exceeds the amount that is available to us by far. Our own attempts to block DTH reactions using the system of Rivas and Ullrich (23) with doses of 1–2 μg IL-10 showed no effect on footpad swelling or epidermal mRNA expression. Alternatively it is possible that the special cellular milieu of the epidermis is necessary for the induction of hapten-specific tolerance in our system.

Our studies also fit with data recently published by Hsieh et al. (25). These authors investigated the capacity of various cytokines to modulate Th1 or Th2 effector cells when included during the primary stimulation with matched APCs. Using naive T cells derived from an MHC class II–restricted, ovalbumin-specific, transgenic T cell receptor mouse, these authors demonstrated that in the absence of additions, a Th0 phenotype producing low amounts of IFN-γ and IL-4 resulted in primary stimulation cultures. In contrast, addition of IL-4 to primary stimulation cultures resulted in a complete polarization to Th2 cells. This effect was independent of the APCs used to initiate T cells. On the contrary, neutralization of endogenous IL-10 during T cell activation markedly increased IFN-γ production and reduced IL-4 and IL-5 production, twisting the immune response towards Th1 development. Although IL-10 by itself did not seem to induce Th2 cell development directly, it might strongly direct development towards the Th2 phenotype by downregulating IFN-γ levels. This effect of IL-10 is dependent on the APC used and supports the notion that APCs together with the local cytokine milieu might determine the phenotype of the responding T cell.

The exact mechanism by which IL-10 induces hapten-specific tolerance in our system remains speculative. Although our in vitro and in vivo data seem to exclude an effect of IL-10 on LC MHC class II expression and although our in vitro data clearly demonstrate that IL-10 converts LC to tolerogenic APCs by inhibiting the upregulation of costimulatory molecules it remains to be proven whether this is the mechanism of IL-10 action in our in vivo system. As a solution for this problem more information about the kind of costimulatory signal affected by IL-10 needs to be collected. Thus far all attempts to characterize the IL-10 effect on the molecular level were without success as neither expression of B7 nor heat-stable antigen or ICAM-1 seem to be affected (9). Nevertheless our studies open up to a whole new spectrum of possible clinical applications of IL-10 in humans and it will be the goal of future studies to determine the molecular details of IL-10 effects in skin.

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