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Reversal of mucosal tolerance by subcutaneous administration of interleukin-12 at the site of attempted sensitization

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SUMMARY

Oral feeding of proteins causes peripheral T-cell tolerance, as revealed by reduced delayed-type hypersensitivity (DTH) reactivity after immunization. This type of tolerance can be due both to passive T-cell anergy and active immunosuppression. Using ovalbumin-fed mice we studied whether putatively immunostimulatory cytokines could break this state of mucosal tolerance. Cytokines were administered locally at the site of attempted sensitization. It was found that neither interleukin-2 (IL-2), interferon-γ (IFN-γ) nor granulocyte–macrophage colony-stimulating factor (GM-CSF) could restore the response to immunization. In contrast, local administration of IL-12 at the site of attempted immunization resulted in full recovery of DTH reactivity. The dichotomy between the two Th1 stimulatory cytokines IFN-γ and IL-12 was also reflected by different effects on ovalbumin-specific antibody isotypes. Although both IFN-γ and IL-12 downregulated serum IgG1-levels in tolerant mice, suggesting decreased ovalbumin-specific Th2 function, only local administration of IL-12 led to increased serum IgG2a levels. These results support the view that potentiation of Th1 effector function is critical for reversal of mucosal tolerance.

INTRODUCTION

Presentation of antigens along mucosal surfaces by oral feeding or intragastric intubation is an effective method to induce peripheral T-cell tolerance. In experimental animal models for allergic contact dermatitis and autoimmune diseases this is readily revealed by reduced delayed-type hypersensitivity (DTH) or autoimmune inflammatory reactivity after subsequent sensitizing attempts.[1–3] In these experimental models mucosal tolerance was found to be persistent, and cutaneous contacts in orally pretreated animals led to further reduced skin reactivity, thus boosting the state of tolerance.[4]

Two mechanisms are currently thought to play important roles in oral tolerance, i.e. clonal anergy and active immune suppression. Clonal anergy is generally favoured by high-dose antigen feeding, whereas active immune suppression may result from low-dose antigen feeding.[2,3,5–7] In the latter models orally induced tolerance was found to be transferable to naive recipients by $CD8^+$ T cells.[1,8] These regulatory cells might exert their suppressive effects via the secretion of distinct cytokines, such as transforming growth factor-β (TGF-β), interleukin-4 (IL-4) and IL-10, and thus would belong to the so-called type 2 T-cell subset(s).[3,5,9–11]

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Antigenic contacts along the mucosal surfaces preferentially induce type 2 T-cell responses. This preference may be related to several factors, including local abundance of IL-4-releasing mast cells[3,12,13] or distinct immunoregulatory hormones,[14] and the tendency of Th0 lymphocytes, when exposed to high, 'anergizing' doses of antigen, to show downregulation of Th1 characteristics and a transition to a Th2-like phenotype.[15–17] Thus, after antigen feeding antigen-specific Th2 cells may develop, which generally do not mediate delayed-type skin hypersensitivity reactions but rather facilitate antigen-specific B-cell differentiation towards the production of distinct antibody isotypes, such as IgG1 and IgA in mice.[5,12,18] These antibodies are optimal in favouring non-inflammatory defence mechanisms within the mucosal tissues to prevent the entry of exogenous microorganisms, and to facilitate their expulsion.[5,18] Although mucosa-derived T cells preferentially recirculate between the mucosa-associated lymphoid tissues, they may also enter peripheral skin-draining lymph nodes since differences in expression of tissue-specific homing receptors on lymphoid cells are gradual rather than absolute.[2,3,6,19] Within peripheral lymph nodes, type 2 T cells, after subsequent skin contacts with the antigen, may be triggered to release their characteristic cytokines, thereby preventing the differentiation of locally residing naive, antigen-specific $CD4^+$ T cells into Th1 effector cells.

If the above outlined hypothesis holds true, then reversal of this state of tolerance might be achieved by changing the local predominant cytokine profile within the lymph nodes draining

the site of attempted sensitization. Recently data have accumulated that IL-12 could have such an effect. IL-12 is a cytokine produced by macrophages and B cells that acts, in general, to promote cell-mediated immunity.[20,21] IL-12 enhances cytotoxic T lymphocyte (CTL) and natural killer (NK) cell cytotoxic activities and interferon-γ (IFN-γ) production.[22] The present evidence suggests that differentiation of Th0 cells towards Th1 or Th2 effector cell functions is primarily regulated by the balance of IL-12 and IL-4 early during the immune response.[21,23] IL-12 is also a co-stimulatory factor for differentiated Th1 cells and required for optimal proliferation and IFN-γ production by Th1 cells in response to antigens.[24,25] The studies reported here were undertaken to determine whether mucosal tolerance might be broken by procedures to increase type 1 effector T-cell function within antigen-draining lymph nodes, so as to interfere with putative type 2 regulatory activities.

MATERIALS AND METHODS

Animals

Inbred male and female BALB/c mice were bred at the Department of Experimental Medicine (Free University, Amsterdam). Mice of either sex were equally divided among experimental groups and were used at the age of 8–14 weeks.

Cytokines

Interferon-γ (rmIFN-γ, 3000 U/ml) was purified from supernatants from CHO-Ki cells that were stably transfected with the murine IFN-γ gene (a kind gift of Dr N. Arai, DNAX, Palo Alto, CA). Initial experiments with granulocyte–macrophage granulocyte-stimulating factor (rmGM-CSF) employed a crude preparation obtained from Dr W. S. Walker, Memphis, TN (600 U/ml), whereas in later experiments rmGM-CSF was used obtained from Behring Werke AG (Marburg, Germany; specific activity $2{\cdot}4 \times 10^7$ U/mg). Interleukin-2 (rhIL-2; 5×10^6 U/ml) was obtained from Eurocetus BV (Amsterdam, the Netherlands). Interleukin-12 (rmIL-12; specific activity 8×10^6 U/mg) was kindly donated by Dr Sypek (Genetics Institute, Cambridge, MA). The cytokines were administered in 30 μl saline as described below.

Induction and assessment of oral tolerance

Tolerance to ovalbumin (OVA; grade V, Sigma, St Louis, MI) was induced by three intragastric feedings with 10 mg OVA in 0·2 ml saline at days -13, -10, and -7. OVA was fed under light ether anaesthesia, via the mouth into the stomach by a flexible tube (1-mm diameter) connected to a 1-ml syringe. Control mice received 0·2 ml saline following the same procedure. At day 0 immunization attempts were made by subcutaneous (s.c.) injection of 50 μg OVA admixed with 30 nmol dimethyldioctadyl ammonium bromide (DDA; Tramedico BV, Weesp, the Netherlands) in 50 μl saline into the left footpad. DDA is now routinely used as an alternative to FCA because it shows very low toxicity.[26] Subsequently cytokines were administered s.c. at the same sites by daily injections at days 1–4. At day 7 mice were challenged by s.c. administration of 10 μg OVA in 30 μl saline into the left ear. Delayed-type hypersensitivity was determined after 24, 48 and 72 hr by measuring differences in ear swelling between left and right (control) ears with an engineers micrometer (Mitutoyo, MFG, Tokyo, Japan). The non-specific increase in ear thickness in non-immunized control groups ranged from 0·005 to 0·025 mm and was routinely substracted from the ear-swelling responses in both control and experimental groups. Control groups consisting of non-fed, immunized (non-cytokine and cytokine treated) mice were included. Each experimental group consisted of six to seven animals and was tested in at least two independent experiments. Control groups consisted of five to eight animals per group. These were tested in eight independent experiments. Ear-swelling responses in the experimental groups are related to those in immunized non-fed, non-cytokine-treated mice and expressed as mean relative ear swelling $\pm$ SD.

Detection of ovalbumin-specific IgG1 and IgG2a antibodies in serum

Levels of OVA-specific IgG1 and IgG2a antibodies were assessed in sera from individual mice, collected 1 week after DTH measurements. Under ether anaesthesia, cardiac punctions were performed with a 1-ml syringe and a 0·6-mm needle. Approximately 0·5–1 ml blood was obtained from each mouse. The blood was placed at room temperature for 1 hr and kept overnight at 4°. Blood clots were removed and sera were stored at $-80°$. Levels of OVA-specific IgG1 and IgG2a antibodies were then determined by enzyme-linked immunosorbent assay (ELISA). Briefly, PVC 96-well ELISA plates (Nunc maxisorp. A/S. Kamstrup, Denmark) were coated with 100 μl of 10 μg OVA/ml coating buffer (1·59 gm Na_2CO_3, 2·93 gm $NaHCO_3$ per litre, pH 9·6) and incubated overnight at 4°. The next day plates were washed once in phosphate-buffered saline (PBS)/0·05% Tween 20 followed by a blocking step with PBS/4% bovine serum albumin (BSA) solution (100 μl/well) for 1 hr at room temperature. Serial dilutions of mouse sera were then incubated for 2 hr at room temperature, after which the plates were washed twice with PBS/0·05% Tween 20. Isotype-specific anti-ovalbumin titres were determined with biotinylated rabbit anti-mouse IgG1 and IgG2a preparations (Zymed, San Francisco, CA; dilutions 1:3000 and 1:8000 respectively). After a 1 hr incubation at room temperature, the plates were washed three times in PBS/0·05% Tween 20, and incubated for 1 hr (room temperature) with peroxidase-conjugated streptavidine (dilution 1:1000; DAKO, A/S, Denmark). After washing in PBS/0·05% Tween 20, ortho-phenylenediamine (Dako, A/S. Denmark) OPD solution (2 mg/7·5 ml PBS) was added to the wells and the reactions were stopped with 1 N H_2SO_4 after 10 min. OD_{490} values were measured using a Titertek Multiscan (Titertek, ICN Biomedicals GmBH, Costa Mesa, CA). Antibody titres were recorded as the highest serum dilution still giving a positive reaction (defined and standardized by the use of positive control sera).

Statistics

P values for the differences between treatment and control groups were determined by the independent sample t-test (two tailed). $P < 0{\cdot}05$ was considered to be statistically significant.

RESULTS

Induction of mucosal tolerance for ovalbumin

Induction of ovalbumin-specific oral tolerance has been widely used as an experimental model in mice.[6,7,8,27] Before initiating

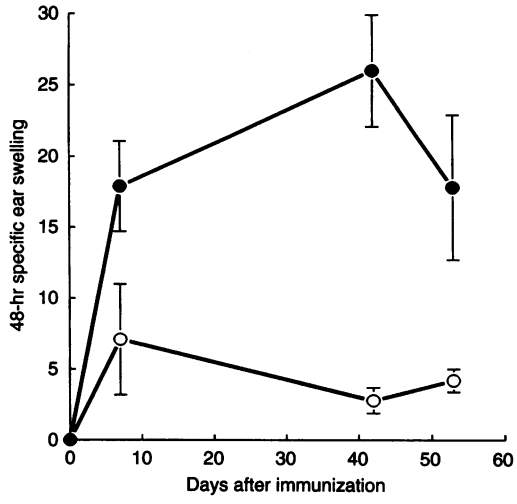

Figure 1. Persistence of oral tolerance to ovalbumin (OVA) after repeated skin challenge. OVA-fed (○) and control mice (●) were immunized with OVA/DDA at day 0 and challenged in the ears at days 7, 40 and 53. Mean ± SD of 48 hr OVA-specific ear swelling responses are given in mm × 10^2.

studies on tolerance reversal by cytokines, we first checked the reproducibility of the model in our hands. Mice were fed three times with 10 mg OVA as described in the Materials and Methods section, and immunization attempts with OVA/DDA were made 1 week after the last feeding. Immunization of control, non-fed mice led to strong DTH responses (mean specific ear swelling at 48 hr 0·11 ± 0·04 mm; $n = 56$). In contrast, previous feeding with OVA led to strongly reduced DTH responsiveness after cutaneous immunization (mean specific ear swelling at 48 hr 0·04 ± 0·02 mm, $n = 56$; $P < 0.005$ as compared with non-fed immunized mice), resulting on average in about 60% reduction of the DTH responses (range 45–75%). This state of tolerance persisted for at least 7 weeks, as shown by repeated challenging with OVA at days 42 and 53 (Fig. 1).

Effect of local administration of cytokines on the development of DTH in orally tolerized mice

Using this model, we next investigated the possibility of restoring the capacity to mount DTH responsiveness by local administration of distinct cytokines at the site of attempted sensitization. Figure 2(a) shows that subcutaneous injections with different dosages of IL-2 up to 30 000 U per day during the first 4 days after the immunization attempt failed to overcome the state of tolerance. Also, repeated injections with low-dose rGM-CSF (crude preparation) or high-dose rGM-CSF (purified recombinant protein) did not lead to detectable restoration of DTH reactivity. We then focused on testing the Th1-stimulating cytokines IFN-γ and IL- 12. The results of the latter experiments are also shown in Fig. 2(a). Local s.c. administration of dosages of IFN-γ up to 3000 U/ml (100 U per site per day) failed to break ovalbumin-induced oral tolerance. In contrast, the capacity to mount DTH responsiveness was fully restored by local administration of 200 U or 800 U of IL-12 during the immunizing attempt. Restoration of T effector cell function was long lasting, as proven by undiminished ovalbumin-specific DTH reactivity revealed by repeated challenging at day 42 (data not shown).

In additional experiments it was confirmed that IL-12 is also unique in its capacity to potentiate DTH responses to OVA in non-fed, immunized mice upon local administration (Fig. 2b).

Effect of local administration of IL-12 or IFN-γ on the development of ovalbumin-specific IgG1 and IgG2a antibodies in orally tolerized mice

Since IL-12 was found to be unique in its tolerance-reversing capacity, we wondered whether the failure of IFN-γ to display a similar capacity was related to different effects on Th1 and Th2 functions. Since in mice Th1 cells facilitate the development of specific antibodies of distinct isotypes including IgG2a,[5,12] we determined the development of ovalbumin-specific IgG2a antibodies in the sera. In addition, to study putative antagonistic effects on Th2 helper function, we determined ovalbumin-specific IgG1 serum titres. Orally tolerized mice

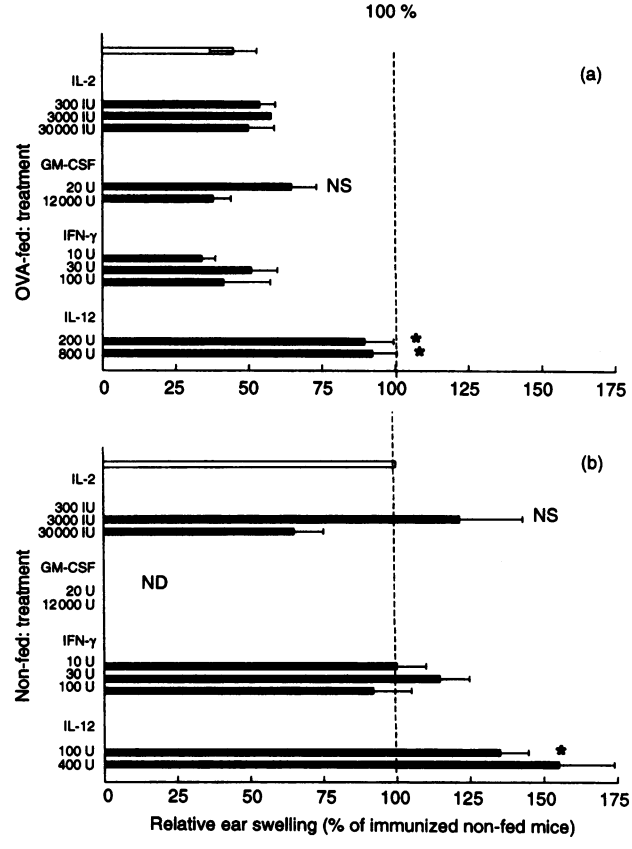

Figure 2. Effects of local cytokine administration on DTH reactivity to OVA in tolerant and normal immunized mice. Cytokines were administered by four daily injections of the indicated dosages at the immunization site in OVA-fed (a) or non-fed (b) mice. Mean specific ear swelling in non-fed mice: 0·10 ± 0·01 mm (100%) and in OVA fed mice: 0·04 ± 0·01 mm. *Reactions significantly increased in tolerized and cytokine-treated mice as compared with tolerized, non-cytokine-treated mice (a; $P < 0.005$), respectively in non-fed, cytokine-treated mice as compared with non-fed immunized mice (b; $P < 0.05$).

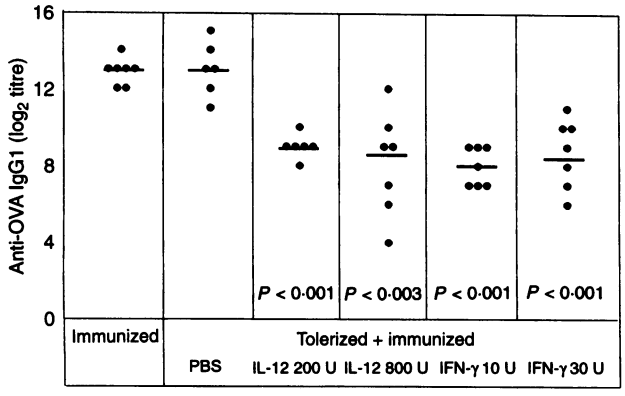

Figure 3. Development of ovalbumin-specific IgG1 serum antibodies in orally tolerized mice treated with different dosages of IFN-γ and IL-12 after immunization. Each dot represents the $\log_2$ titre of OVA-specific antibodies in individual mice, assessed by ELISA, 14 days after immunization. For statistical analyses cytokine-treated mice were compared with PBS-treated tolerant mice. Lines represent the mean titre for each group.

appeared to mount similar titres of both IgG1 and IgG2a antibodies upon immunization with OVA in DDA as compared with control mice (Figs 3 and 4). Figure 4 shows that local treatment with IL-12, but not with IFN-γ, led to a small but significant increase of ovalbumin-reactive IgG2a antibodies, as compared with both normal immunized and tolerant control groups respectively. Noticeably, both IFN-γ and IL-12 treatments resulted in markedly decreased titres of Th2-dependent ovalbumin-specific IgG1 antibodies (Fig. 3).

DISCUSSION

The primary aim of this study was to investigate whether one or more of the cytokines currently known for their strong capacities to augment Th1 effector cell functions, i.e. IL-2, IL-12, IFN-γ and GM-CSF, could break the state of tolerance induced by previous feedings with ovalbumin. The present results make it clear that only IL-12 exerts this function. The finding that GM-CSF did not restore immune reactivity suggests that decreased local antigen-presenting cell (APC) function within the lymph nodes draining the site of antigen administration is not a critical factor in the failure of residing antigen-specific Th0 cells to give rise to Th1 effector cell progeny. This result is in line with our earlier findings in mice tolerized by feeding with the contact sensitizer oxazolone, showing that APC from allergen-fed, tolerant mice were fully capable of initiating DTH.[28] The finding that locally increased levels of IL-2 within the antigen-reactive lymph nodes also failed to restore effector T-cell development, argues against high-dose antigen-induced anergy as being an important factor in this tolerance model. In *in vitro* models, IL-2 was found to be effective in breaking T-cell anergy.[27]

In contrast, local administration of the pivotal Th1 cytokine IL-12 fully reversed the capacity of tolerized mice to develop cell-mediated immune function. This finding supports the view that release of type 2 cytokines within antigen-stimulated peripheral lymph nodes can play a role in orally induced ovalbumin tolerance. Most likely, the producer cells stem from the mucosal lymphoid tissues originally exposed to the antigen. Interleukin-12 could exert its major tolerance breaking activity at least at two different levels, i.e. by interfering with type 2 regulatory cell function, for instance by inhibiting biological functions of IL-4 and IL-10,[21,22,29] and by providing costimulatory signals for Th1 development.[24,25] Our data on ovalbumin-specific antibody profiles reflecting Th1 and Th2 functions respectively, suggest that at least potentiation of Th1 effector function is critical for reversal of mucosal tolerance. First, we observed that in contrast to IL-12, the Th1 cytokine IFN-γ was unable to reverse tolerance. Noticeably, by ELISA, IFN-γ was similarly effective as IL-12 in reducing the production of Th2-related IgG1 antibodies. Thus, within the antigen-reactive draining lymph nodes, increased levels of both IL-12 and IFN-γ led to interference with Th2 function. In contrast, significant augmentation of antigen-specific Th1 function was only obtained by administration of IL-12, as reflected by the release of high amounts of ovalbumin-specific IgG2a into the circulation. Interestingly, such high levels of IgG2a antibodies were not observed after regular immunization of control, non-tolerized mice. This would suggest that peripheral Th1 effector cell function develops independently from local antigen-specific Th1 help for antibody development. A similar dichotomy between IL-12 and IFN-γ activities has been reported in experimental murine candidiasis, in which IL-12 rather than IFN-γ production reflected Th1 differentiation.[30]

Recently, using normal, non-tolerized mice, Maguire *et al.*[31] reported that local administration of IL-12 at the site of application of the contact sensitizer dinitro-chlorobenzene, strongly augmented the development of contact hypersensitivity. Our results also show that in the ovalbumin model, local administration of IL-12 enhances immunization efficacy to OVA as reflected by increased DTH responses after local administration during immunization (Fig. 2b). This supports the hypothesis of Maguire *et al.*[31] that local release of type 2 cytokines also contributes to the regulation of T effector function in normal immunized mice. Thus IL-12 represents a potentially effective immune adjuvant for cell-mediated immune functions. Our present results show that the latter

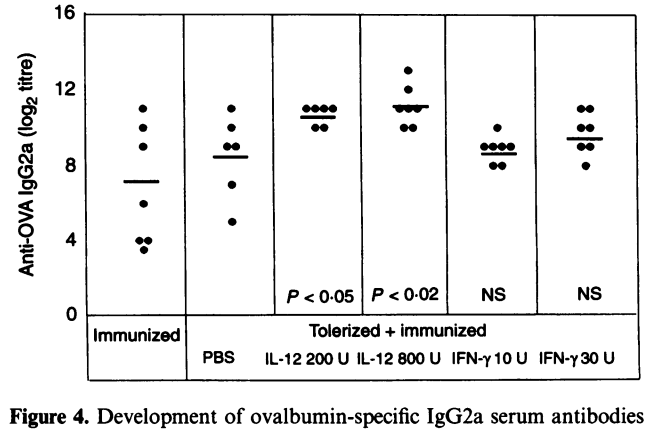

Figure 4. Development of ovalbumin-specific IgG2a serum antibodies in orally tolerized mice treated with different dosages of IFN-γ and IL-12 after immunization. Each dot represents the $\log_2$ titre of OVA-specific antibodies in individual mice, assessed by ELISA, 14 days after immunization. For statistical analyses cytokine-treated mice were compared with PBS-treated tolerant mice. Lines represent the mean titre for each group.

conclusion holds even under conditions of mucosal tolerance. The latter notion may be of particular relevance to clinical cancer involving tumours arising from mucosal surfaces. Both anergic tumour-infiltrating lymphocytes and tumour-infiltrating lymphocytes belonging to the Th2 subset have been isolated in several tumour types.[32–34] Thus, introducing IL-12 into tumour vaccines, either by simultaneous administration by local depot systems, or by gene transfection,[35] provides a promising option for enhancement of vaccine efficacy and reversal of tumour antigen-specific tolerance that might have been induced at an early stage of tumour growth.

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