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Bystander suppression of the immune response to human serum albumin in rats fed ovalbumin

A. DAHLMAN-HÖGLUND,* U. DAHLGREN,*† S. AHLSTEDT,* L. Å. HANSON* & E. TELEMO*
**Department of Clinical Immunology, and †Department of Endodontics/Oral Diagnosis, University of Göteborg, Sweden*

SUMMARY

Bystander suppression of delayed-type hypersensitivity (DTH) and the antibody response to human serum albumin (HSA) were studied in young normal rats and in young rats made partially tolerant to ovalbumin (OVA) by feeding an OVA-containing diet for 4 weeks from weaning. At 2 months of age, the animals were intracutaneously immunized with a mixture of OVA and HSA in Freund's complete adjuvant (FCA) at one site of the back, or separately at two different sites on the back. All rats made orally tolerant to OVA showed a significantly reduced IgE and IgG anti-OVA antibody production and DTH response to OVA, compared to the controls. OVA-fed rats subsequently immunized with a mixture of OVA + HSA had significantly lower IgE and DTH responses to HSA than the controls. When rats were immunized with OVA and HSA at two different sites, however, there was no difference in the response to HSA between the OVA-fed rats and the control rats, which rules out the possibility of shared epitopes between the antigens. Ear-challenge with the mixture of OVA + HSA gave a significantly lower DTH reaction in the tolerant rats immunized with a mixture of the antigens, compared to the control rats. However, suppression of the DTH reaction was not seen when tolerant and control rats were immunized with HSA alone and challenged with the mixture of OVA + HSA in one ear. These results present evidence that young rats orally tolerant to one antigen show a suppressed T-cell and antibody response to an unrelated antigen, provided that the two antigens are given in a mixture during the inductive phase. There was no evidence for bystander suppression of the T-cell response at the effector site.

INTRODUCTION

Feeding soluble antigens to neonatal and adult animals often leads to an antigen-specific state of unresponsiveness, referred to as oral tolerance. The suppression may act on both antibody responses and T-cell reactivity.[1–4] Clonal anergy and/or active cell-mediated suppression are suggested to be the mechanisms behind tolerance to protein antigens administered orally in experimental animals.[5,6] High doses of fed antigen is claimed to favour clonal anergy, whereas lower doses may favour active suppression.[7,8] Clonal anergy is thought to occur when antigens are presented by non-professional antigen-presenting cells (APC), lacking critical ligands for helper T-cell activation such as CD80, CD40 or lymphocyte function-associated antigen-1 (LFA-1).[9] Active suppression has been shown to be mediated by specialized $CD8^+$ T-suppressor cells that are triggered through an antigen-specific mechanism, but provide their effect via antigen non-specific suppressive factors such as certain cytokines.[6,10] The cytokines responsible for suppression are interferon-γ (IFN-γ), transforming growth factor-β (TGF-β), and interleukin-10 (IL-10), which are produced by the $CD8^+$ T-suppressor cells.[11] The production of antigen non-specific suppressive cytokines can result in the suppression of an *in vitro* and *in vivo* response to an unrelated locally present antigen, so called bystander suppression.[12,13]

Oral administration of myelin basic proteins (MBP) to rats has been shown to induce MBP-specific $CD8^+$ T cells producing TGF-β, preventing the outbreak of autoimmune encephalomyelitis.[6] Antigen-specific $CD8^+$ T cells have also been shown to transfer active suppression from tolerized animals to naive recipients.[10] We wanted to study *in vivo* T-cell reactivity and antibody response to two exogenous antigens, human serum albumin (HSA) and ovalbumin (OVA), in young rats orally tolerant to OVA, after different routes of immunization with OVA and HSA. This was to find out whether or not there was an active suppression both at the site of immunization (inductive site) and at the site of a delayed-type hypersensitivity (DTH) reaction (effector site).

MATERIALS AND METHODS

Animals

Three-week-old Sprague–Dawley rats were bred in our own animal house facilities (Dept. of Clinical Immunology, Göteborg, Sweden), in a temperature- and light-controlled

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Correspondence: Dr A. Dahlman-Höglund, Department of Clinical Immunology, Guldhedsgatan 10A, 5-41346 Göteborg, Sweden.

environment, with free access to food and water. In each experiment, rats were matched for sex and age.

Rat diet

The standard diet was based on cereals, fish, soy and yeast, with a dry weight protein content of 24% (R3; Ewos, Södertälje, Sweden). The OVA-diet consisted of the standard diet but half of the protein content had been replaced with equal parts of egg protein and milk whey protein.[14]

Experimental procedure

Experiment 1, immunization with a mixture of two antigens at one site. The rats were weaned at 21 days of age onto an OVA-containing diet fed *ad libitum* for 4 weeks. The intake of OVA from this diet was estimated to be 0·8 g/rat/day.[14] Control rats were weaned onto the standard diet. At 8 weeks of age the rats were given an intracutaneous injection of 100 μl of OVA + HSA [Sigma, St Louis, MO; 20 mg/ml of each protein in a mixture of phosphate-buffered saline (PBS) and an equal volume of Freund's complete adjuvant (FCA)] at one site on the back (Table 1). The DTH reaction was tested 2 weeks later, by an intradermal injection in the ear with 20 μl of the appropriate antigen solution (2·5 mg/ml) in PBS. Rats in group 1 were challenged with OVA + HSA in one ear (Table 1); rats in group 2 were challenged with OVA in one ear and HSA in the other ear; and rats in group 3 were challenged with HSA in one ear. The increase in ear thickness was measured 24 hr later with an Oditest (Kröplin, Hessen, Germany).

Experiment 2, immunization with two antigens at two different sites. The rats were fed in the same way as in experiment 1. At 8 weeks of age the rats were divided into five groups, with equal numbers of OVA-fed and control rats in each group. Groups 1–3 were identical with experiment 1, but two extra groups (4 and 5) were included. The rats in group 4 were immunized with OVA at one site on the back, and the rats in group 5 were immunized with OVA and HSA at two different sites on the back (Table 1). Two weeks later, they were challenged intradermally in one or two ears, as in experiment 1. Rats in groups 3 and 4 were challenged with HSA in one ear and rats in group 5 were challenged with OVA in one ear and HSA in the other ear.

Experiment 3, control experiment. The rats in this experiment were fed in the same way as in experiment 1, but they were all immunized with 100 μl HSA only, in FCA, at one site of the back (Table 1). Two weeks later they were tested for DTH reactions as described for experiment 1.

Collection of serum

After measurements of the increase in ear thickness, all rats were killed and blood samples were taken from the tail and stored at −20°, until analysed.

Antibody determinations

IgG and IgE antibodies against OVA and HSA in serum were analysed with an enzyme-linked immunosorbent assay (ELISA), as described elsewhere.[15] For IgE antibody measurement, a mouse monoclonal anti-rat IgE–biotin conjugate (MCA 193B; Serotec, Oxford, UK), diluted 1/1000 in PBS–Tween, and a streptavidine–alkaline–phosphatase conjugate (DAKO A/S, Glostrup, Denmark), diluted 1/2000 in PBS–Tween, were used. For IgG antibody quantification, rabbit anti-rat IgG (Zymed Laboratories, Inc., San Francisco, CA), diluted 1/10 000 in PBS–Tween, and goat anti-rabbit IgG–alkaline–phosphatase conjugated antibody (Sigma), diluted 1/10 000 in PBS–Tween, were used. The specificity of the antibody reagents was tested extensively against rat myeloma proteins (PharMingen, San Diego, CA) and polyclonal rat IgG.

All values were calculated and compared with a hyperimmune serum with the help of a computer program, and are expressed as arbitrary ELISA units for each class in the diagrams.

Statistics

The data were tested for statistical significance by the Mann–Whitney *U*-test.

RESULTS

Experiment 1, immunization of a mixture of two antigens at one site

The rats that had been eating the OVA-containing diet *ad libitum* and immunized with a mixture of OVA and HSA (groups 1 and 2) showed a significantly lower IgE and IgG antibody response against OVA ($P < 0{\cdot}0001$ for both isotypes) compared with the control rats (Fig. 1). They also had a significantly reduced IgE antibody response to HSA ($P < 0{\cdot}0001$), but there was no significant difference in the levels of IgG anti-HSA antibodies between the two groups. The DTH reaction of the rats immunized with the mixture of OVA + HSA is shown in Fig. 2. A suppressed DTH response to OVA were observed in all rats fed OVA and challenged with OVA or OVA + HSA. Rats made tolerant to OVA (group 2)

Table 1. Experimental protocol

Experiment	1	2	3	4	5	6
Group no.	1	2	3	4	5	6
Immunization	OVA + HSA	OVA + HSA	OVA + HSA	OVA	OVA/HSA	HSA
Challenge	OVA + HSA	OVA/HSA	HSA	HSA	OVA/HSA	OVA + HSA/HSA

OVA + HSA = mixed antigens used for immunization at one site on the back in FCA, or mixed antigens in PBS used for challenge in one ear.

OVA/HSA = each antigen used for immunization at two different sites on the back in FCA, or given as challenge with OVA in one ear and HSA in the other ear.

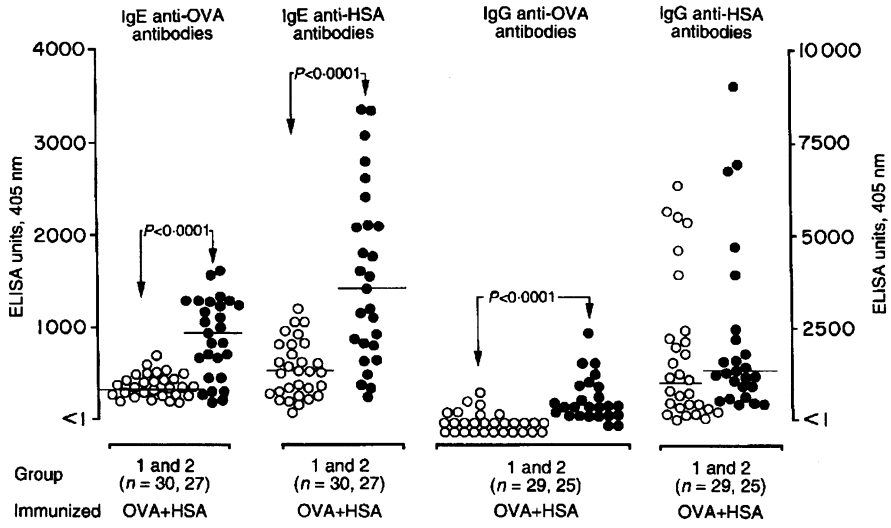

Figure 1. IgE and IgG antibodies in serum against OVA and HSA recorded by ELISA in 10-week-old rats, 2 weeks after intracutaneous immunization with a mixture of OVA and HSA in FCA 2 weeks earlier. (○) Rats made tolerant by feeding an OVA-containing diet from weaning onwards. (●) Control rats fed a non-OVA-containing diet from weaning. Each symbol represents one rat. Groups 1 and 2 are combined in the antibody analyses.

also had a significantly lower DTH reaction when challenged with HSA alone ($P = 0{\cdot}0005$), compared with the control rats.

Experiment 2, immunization of two antigens at two different sites

To determine if bystander suppression of the response to HSA was only seen when OVA-tolerant rats were immunized with a mixture of OVA + HSA in FCA at the same site, as in experiment 1, the experiment was repeated but the rats were immunized with OVA and HSA separately at two different sites on the back. As seen in Fig. 3a, the OVA-tolerant rats immunized with OVA + HSA at the same site showed a significantly reduced IgE antibody response to HSA ($P = 0{\cdot}0045$) compared with the non-tolerant control rats, while there was no significant difference in IgE antibody response to HSA between the OVA-tolerant and the control rats immunized with OVA and HSA at different sites. There was no significant difference in the levels of IgG anti-HSA antibodies between the OVA-tolerant rats immunized with OVA + HSA at the same site or at separate sites and the control rats (data not shown). In addition, the DTH response to HSA in the OVA-tolerant rats immunized with the mixture of OVA + HSA was significantly lower ($P = 0{\cdot}017$) than the non-tolerant control rats. In contrast, there was no significant difference in DTH response to HSA between the OVA-tolerant and the control rats immunized with OVA and HSA at different sites (Fig. 4). Again, the OVA-tolerant rats showed a significantly lower DTH, IgE and IgG antibody response against OVA ($P = 0{\cdot}004$, $P < 0{\cdot}002$) compared to the control rats (Figs 4 and 3b).

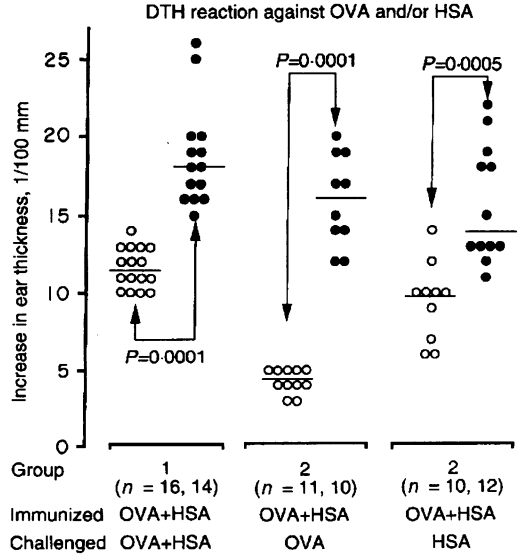

Figure 2. DTH reaction against OVA and HSA measured as increase in ear thickness in 10-week-old rats. All rats had been immunized intracutaneously with a mixture of OVA and HSA in FCA 2 weeks earlier. (○) Rats made tolerant by feeding with an OVA-containing diet from weaning. (●) Control rats fed a non-OVA-containing diet from weaning on. Each symbol represents one rat.

Experiment 3, control experiment

As a control experiment both OVA-tolerant and control rats were immunized with only HSA in FCA. There were no differences in either antibody or DTH responses to HSA between the OVA-tolerant and the control rats (Fig. 5). Challenge of these rats with OVA + HSA in one ear and HSA in the other ear, showed no difference in DTH reaction between the two groups.

DISCUSSION

The present study was undertaken to investigate whether orally

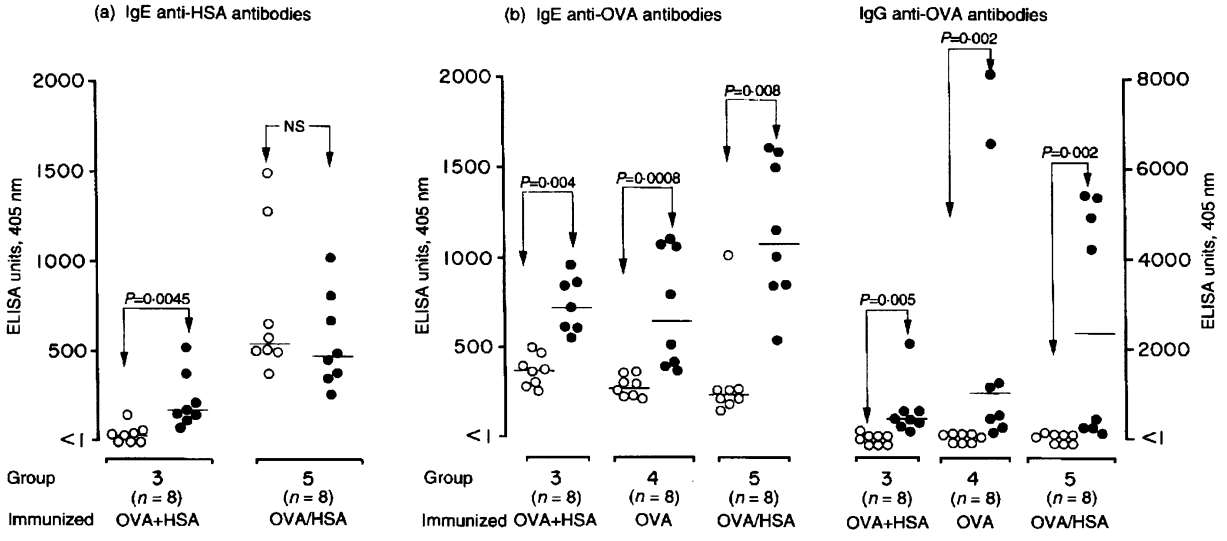

Figure 3. (a) IgE antibodies in serum against HSA recorded by ELISA in 10-week-old rats, 2 weeks after intracutaneous immunization with a mixture of OVA and HSA, or separately at two different sites on the back, in FCA. (b) IgE and IgG antibodies in serum against OVA recorded by ELISA in 10-week-old rats. The rats had been immunized intracutaneously with OVA, a mixture of OVA and HSA, or with OVA and HSA, separately at two different sites on the back, in FCA 2 weeks earlier. (○) Rats made tolerant by feeding an OVA-containing diet from weaning. (●) Control rats fed a non-OVA-containing diet from weaning. Each symbol represents one rat. NS, not significant.

induced tolerance to a specific heterologous antigen alters the response to an unrelated antigen given simultaneously either at the induction site or at the effector site of the immune response. The results showed evidence for bystander suppression of the DTH and IgE, but not the IgG, antibody response to HSA in the rats orally tolerant to OVA. The bystander suppression was only seen in OVA-tolerant animals, when the two antigens were injected at same time and site in FCA. Thus, when the rats were immunized with OVA and HSA at different sites, a normal antibody response to HSA was observed in both controls and in rats fed OVA, ruling out the possibility of shared epitopes between OVA and HSA. Similarly, Miller *et al.*[12] have shown *in vivo* bystander suppression in rats tolerant to MBP, when the secondary antigen was injected subcutaneously in PBS 8 hr after

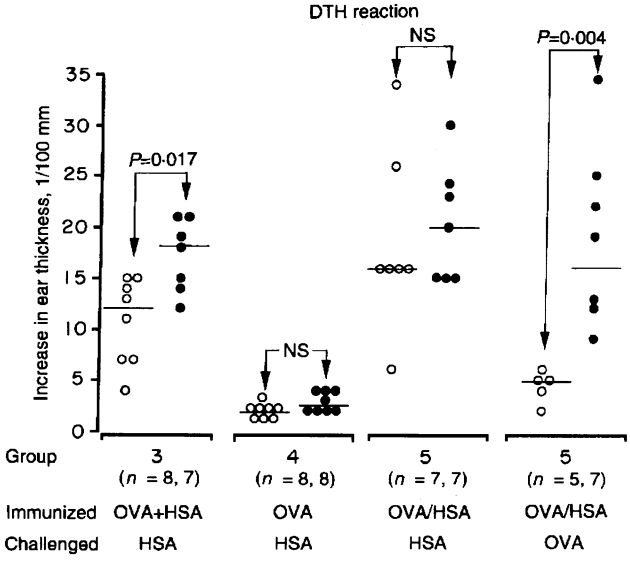

Figure 4. DTH reactions against OVA and HSA measured as ear thickness in 10-week-old rats. The rats had been immunized intracutaneously with OVA, a mixture of OVA and HSA, or with OVA and HSA, separately at two different sites on the back, in FCA 2 weeks earlier. (○) Rats made tolerant by feeding an OVA-containing diet from weaning. (●) Control rats fed a non-OVA-containing diet from weaning. Each symbol represents one rat.

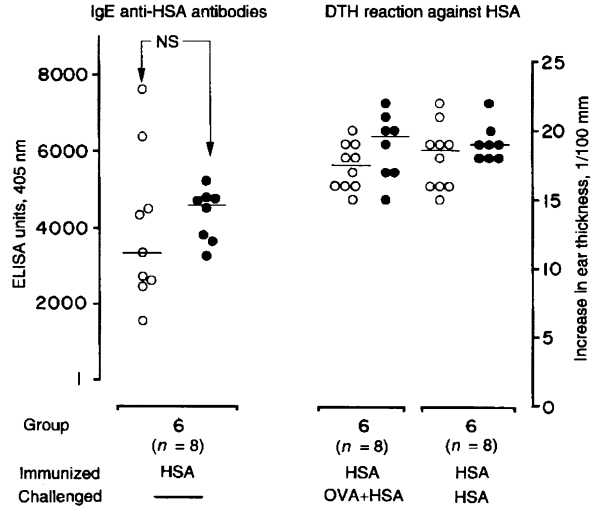

Figure 5. IgE antibodies in serum against HSA recorded by ELISA, and DTH against OVA and HSA measured as ear thickness in 10-week-old rats. All rats had been immunized intracutaneously with HSA in FCA 2 weeks earlier. (○) Rats made tolerant by feeding an OVA-containing diet from weaning. (●) Control rats fed a non-OVA-containing diet from weaning. Each symbol represents one rat.

the primary immunization with antigen in FCA in the same footpad. Together, these results imply that a bystander suppression to an unrelated protein is possible only if the antigen is injected at the same site as, but not necessarily simultaneously with, the primary antigen to which the rat is tolerant.

Immunological tolerance may occur through different regulatory mechanisms depending on how an antigen is introduced into the animals.[4,16,17] The stage of maturation of the intestinal immune system seems to be important for the outcome of the response to orally applied antigens.[18] Feeding protein antigen to mice before 5 days of age primes for a subsequent elevated immune response to the antigen. Miller *et al.*[19] also showed recently that neonatal rats fed MBP became primed, while rats fed MBP at 4 weeks of age became tolerant. Interestingly, the timing of these events coincided with the vesicular expression of rat class II antigen in the epithelial cells of the small intestine.[20] The most important factor in the development of active suppression therefore seems to be the age of the animals rather than the dose of fed antigen. This is supported by the active suppression seen in the present investigation, despite an estimated daily intake of 0·8 g OVA.[14] In addition, recent results from our laboratory (Lundin *et al.*, unpublished data) show that T cells from rats fed the OVA-containing diet, during 4 weeks from 6–7 weeks of age, could transfer their OVA tolerance to naive recipients.

In orally/aerosol tolerized adult rats it has been found that they have both reduced T-helper type-2 (Th2) and T-helper type-1 cell (Th1) function. These animals showed secretion of IFN-γ and IL-2 from both $CD4^+$ and $CD8^+$ cells, but IFN-γ was restricted to enriched $CD8^+$ cells.[10,13] The mechanism behind active suppression of the $CD4^+$ Th2 response has been suggested to be due to antigen-specific IFN-γ-producing $CD8^+$ T cells, which recently have been shown to express $\delta\gamma$ T-cell receptor.[21] These T cells are in turn activated by IL-2-producing $CD4^+$ cells, hence the necessity for class II expression.[10] Activated $CD8^+$ T cells secrete TGF-β and IL-10, which function as antigen non-specific suppressive factors.[6] This active suppression can be transferred to naive recipients with $CD8^+$ T cells.[22] On the other hand, Burstein & Abbas[23] demonstrated that mice tolerized by intraperitoneal administration of antigen showed an inhibition of Th1 cell activity and thus of IFN-γ and IL-2 secretion, but no inhibition of Th2 cells secreting IL-4, which induce IgE production. The OVA-fed rats in this study showed suppression of T- and B-cell reactivity to OVA, involving both Th1 and Th2 cells.

In conclusion, oral tolerance is maintained by antigen-specific cells that can exert bystander suppression of T- and B-cell reactivity to an unrelated non-cross-reactive antigen if a mixture of the two antigens is given during the inductive phase.

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