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Suppression of an established DTH response to ovalbumin in mice by feeding antigen after immunization

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SUMMARY

Experiments were designed to examine whether systemic delayed-type hypersensitivity responses (DTH) to ovalbumin (OVA) can be suppressed when antigen is fed after immunization, and to investigate the immunological mechanisms involved. A single 25 mg feed of OVA given 7 or 14 days after immunization with OVA in complete Freund's adjuvant (CFA) suppressed the DTH response of BDF1 mice, but had no significant effect on the serum IgG antibody response. DTH suppression was greatest when antigen was fed soon after immunization, and became less pronounced as the time interval between feeding and immunization increased. The phenomenon was also demonstrated in mice of the BALB/c strain. Cell transfer experiments suggested that the post-immunization suppression was not due to a population of suppressor cells that have been described previously in association with classical oral tolerance for DTH. We conclude that there are separate and distinct mechanisms for the prevention of induction of DTH by antigen feeding in naive mice and the suppression of expression of DTH in sensitized animals.

INTRODUCTION

The induction of immunological tolerance by the feeding of antigen is well-documented (reviewed by Stokes, 1984; Challacombe & Tomasi, 1987; Mowat, 1987). The biological importance of this phenomenon is suggested by the diversity of systemic immune responses suppressed, and the wide range of antigens which can be used to induce oral tolerance (Stokes, 1984). Since failure of oral tolerance mechanisms may lead to systemic or intestinal hypersensitivity to food antigens (Mowat & Ferguson, 1981), further elucidation of the immunological consequences of feeding antigen could be of potential importance in designing strategies to prevent or treat such hypersensitivity reactions in the gut and other organs.

Most research on this topic has been conducted in immunologically naive animals. Antigen is fed prior to systemic immunization, and suppression of subsequent systemic antibody and DTH responses used as evidence of oral tolerance. Little attention has been paid to the possibility that immune responses in already sensitized animals may be susceptible to orally induced suppression. Early reports suggested that feeding antigen to previously immunized animals enhanced rather than suppressed humoral immune responses (Hanson, *et al.*, 1979; Titus & Chiller, 1981b). However, other workers have shown recently that antibody production in immunized hosts can be down-regulated (Lafont *et al.*, 1982; Bloch *et al.*, 1983; Saklayen *et al.*, 1984).

Manipulation of the cell-mediated limb of systmic immunity by feeding antigen has, to date, been neglected. Therefore, we have examined the effects of feeding tolerogenic doses of antigen on DTH responses of systemically immunized mice.

MATERIALS AND METHODS

Animals

Female BALB/c and BDF1 (C57BL/6J × DBA/2) mice were used throughout. These mice were bred and maintained in the Animal Unit, Western General Hospital, Edinburgh, and were first used at 6–10 weeks of age.

Antigens

Ovalbumin (OVA; Sigma fraction V, Sigma, Poole, Dorset) and human serum albumin (HSA; Sigma fraction V, Sigma) were dissolved in sterile saline before use. Heat-aggregated OVA was prepared by the method of Titus & Chiller (1981a) and stored at −20° before use.

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Abbreviations: CFA, complete Freund's adjuvant; CMI, cell-mediated immunity; 2dGuo, 2-deoxyguanosine; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; HA, haemagglutinin antigen of influenza virus; HSA, human serum albumin; OVA, ovalbumin; Th/DTH, helper/delayed-type hypersensitivity T cell; Ts, suppressor T cell.

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Oral administration of antigen

OVA or HSA was dissolved in sterile saline to a concentration of 125 mg/ml and animals were fed 25 mg via a rigid feeding tube. Control mice in these experiments received 0·2 ml saline. The day of systemic immunization (see below) is referred to as Day 0, and the day of antigen feeding is given relative to this point, with a negative prefix indicating that feeding occurred before immunization.

Immunization and assessment of systemic antibody and DTH responses

Animals were immunized with 100 μg OVA emulsified in 0·05 ml Freund's complete adjuvant (CFA, Bacto H37Ra; Difco Ltd, West Molesey, Surrey) into one rear footpad. Mice were bled under light ether anaeasthesia, 20 days after immunization, and the serum fraction was assayed for IgG antibodies to OVA by an ELISA technique (Lamont, Gordon & Ferguson, 1987).

At 21 days after immunization, the mice were tested for DTH responses by the increment in thickness of the non-immunized footpad 24 hr after intradermal challenge with 100 μg heat-aggregated OVA.

2-Deoxyguanosine

2-Deoxyguanosine (2dGuo; Sigma) was dissolved in sterile distilled water at 5 mg/ml, and given intraperitoneally at a dose of 1 mg daily.

Preparation and transfer of cell suspensions

Spleens were removed from mice and placed in RPMI-1640 (Gibco, Paisley, Renfrewshire). They were cleaned of adherent tissue, minced using dissecting scissors, and gently passed through thin-gauge wire mesh. The resultant cell suspension was allowed to stand for several minutes to allow debris to settle, and then washed three times. Viability, as assessed by trypan blue exclusion, was greater than 80%. Cells were transferred intraperitoneally into recipient mice according to the protocols described below.

Statistics

DTH responses are expressed as the mean ± 1 SD and were compared by Student's *t*-test. Serum antibody responses are expresssed as the geometric mean and range of individual optical density readings, and were compared using the Wilcoxon rank sum test.

Values for percentage suppression were obtained as follows:

$$\% \text{ suppression} = \frac{\text{responses in control group} - \text{responses in tolerant group}}{\text{response in control group}} \times 100.$$

RESULTS

Early time-course of DTH in immunized mice

In order to provide baseline information on the *in vivo* DTH responses after immunization, BDF1 mice were immunized with 100 μg OVA in CFA and tested for DTH, as described above, 2, 7 and 14 days later. Non-immunized mice were used as negative controls. The skin test was negative at 2 days post-immunization, but positive at 7 days (mean increment in footpad thickness 0·12 mm, SD 0·03), and 14 days (mean 0·25 mm, SD 0·12).

Effect of OVA feeding after immunization on systemic immune responses

In previous experiments we have used a single feed of 25 mg of antigen 7 or 14 days before immunization to induce tolerance of antibody and DTH responses (Mowat *et al.*, 1982, 1986). In the present studies we used a similar protocol. Groups of mice were fed saline (Group a), 25 mg OVA (Group b) or, as a specificity control, 25 mg HSA (Group c), 7 days before immunization (i.e. on Day −7). Other mice received a feed of OVA on the day of systemic immunization, Day 0 (Group d), 7 days later (Group e) or 14 days later (Group f). All groups were assessed for serum IgG antibody and DTH responses 3 weeks after immunization, as described above.

As expected, feeding OVA on Day −7 (Group b) resulted in suppression of the DTH response (Fig. 1; $P < 0.01$) compared to group a). The induction of tolerance was antigen specific as a feed of HSA had no effect on the DTH response to OVA (Group c). DTH suppression was also observed, when mice were fed OVA on Day 0 (Group d), Day 7 (Group e) or Day 14 (Group f) ($P < 0.01$ compared to Group a). Thus, orally induced suppression of the DTH response occurred, irrespective of whether OVA was fed 7 days before, at the same time as, or up to 14 days after immunization.

Different effects on serum antibody responses were observed (Table 1). Tolerance was induced in animals fed OVA prior to immunization (Group h, 33% suppression compared to group a, $P < 0.01$), but this was not observed in animals that had been fed OVA 7 days (Group e) or 14 days (Group f) after immunization ($P > 0.05$). The mice which received an OVA feed on the day of immunization (Group d) showed a low level of tolerance (20% suppression; $P < 0.05$).

A less extensive experiment was carried out to examine mice of a different inbred strain (BALB/c) for the phenomenon of post-immunization DTH suppression (Table 2). In these mice, feeding on the same day (Day 0) or 7 days after (Day 7) immunization resulted in suppression of the DTH response (72% suppression, $P < 0.005$, and 52% suppression, $P < 0.02$,

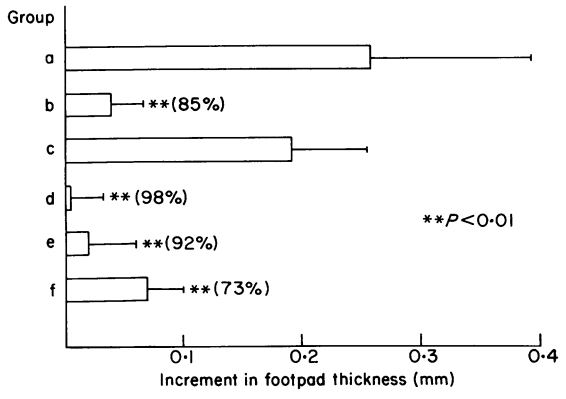

Figure 1. Oral tolerance for DTH responses in BDF1 mice. All groups were immunized on Day 0 and DTH responses were assessed 3 weeks later. Groups are as follows: Group a, saline fed (Day −7); Group b, OVA fed (Day −7); Group c, HSA fed (Day −7); Group d, OVA fed (Day 0); Group e, OVA fed (Day 7); Group f, OVA fed (Day 14). Results shown are mean specific increments in footpad thickness ± 1 SD for groups of six to eight mice.

Table 1. Oral tolerance for serum IgG antibody responses in BDF1 mice

Group	IgG antibody (OD_{405}) Mean	Range
a	0·855	0·811–0·902
b	0·572	0·351–0·711
c	0·891	0·788–1·038
d	0·681	0·520–0·833
e	0·674	0·488–0·916
f	0·826	0·760–0·863

Response were measured by ELISA 20 days post-immunization and the results expressed as the geometric mean and range of individual OD_{405} readings. For description of groups, see legend to Fig. 1.

Table 2. Oral tolerance for DTH and IgG antibody responses in BALB/c mice

		Saline fed Day 0	OVA fed Day 0	OVA fed Day 7
DTH responses (mm)	Mean	0·209	0·058	0·101
	sd	0·083	0·059	0·044
IgG antibody (OD_{405})	Mean	0·406	0·216	0·324
	Range	0·288–0·565	0·096–0·373	0·218–0·534

All groups were immunized on Day 0, and were subsequently fed OVA or saline as indicated. DTH responses were assessed 3 weeks later, and are expressed as mean specific increments in footpads thickness ±1 SD. Antibody responses were measured by ELISA 20 days post-immunization, and are expressed as the geometric mean and range of individual OD_{405} readings. All groups contained six to eight mice.

respectively). There was suppression of the IgG antibody response by feeding on Day 0 (47% suppression, $P=0·02$) but not on Day 7 ($P>0·05$).

Mechanism of suppression of DTH: effect of 2dGuo

Administration of 2dGuo after feeding OVA, and before systemic immunization, prevents the induction of oral tolerance, probably by an effect on the generation of OVA-specific Ts cells (Mowat, 1986). Thus, in an attempt to establish whether a similar population of suppressor cells are responsible for post-immunization suppression by feeding, groups of mice were fed OVA or saline 7 days after immunization, and then received seven daily injections of 1 mg 2dGuo intraperitoneally. Control groups received daily injections of distilled water. DTH responses were assessed 21 days after immunization.

A single feed of 25 mg OVA, 1 week after immunization resulted in 64% suppression of the DTH response (Fig. 2; $P<0·01$ compared to the saline-fed control). There was a similar

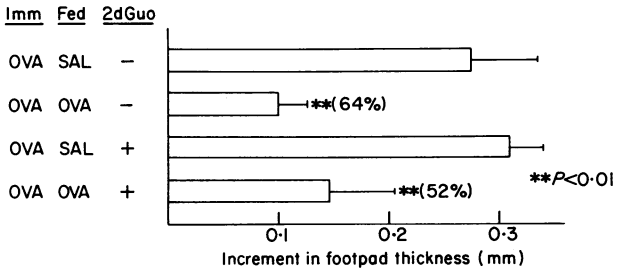

Figure 2. Effect of 2dGuo on orally induced suppression of DTH. All groups were immunized on Day 0, and were fed OVA or saline 7 days later. Two groups subsequently received seven daily injections of 1 mg 2dGuo i.p., while control groups were sham-injected with distilled water. DTH responses were assessed 3 weeks after immunization, and are expressed as mean specific increment in footpad thickness ±1 SD for groups of six to eight BDF1 mice.

degree of suppression in OVA-fed mice which had received 2dGuo (52% suppression; $P<0·01$ compared to saline/2dGuo control). Thus, post-immunization suppression of DTH could not be attributed to a population of 2dGuo-sensitive suppressor cells.

Adoptive transfer of suppression from OVA-immunized and fed mice

Adoptive cell transfer was used as another approach to defining the mechanism of suppression of DTH. Experiments were carried out in order to establish the suppressor properties of spleen cell suspensions at two time-points after antigen feeding.

7 days after OVA feed

Donor mice were immunized with OVA in CFA, and 1 week later were given a feed of 25 mg OVA or saline. Eight mice per group were retained, and skin tested at 3 weeks to confirm that, as in previous experiments, feeding OVA 1 week after immunization resulted in almost complete suppression of the DTH response (97% suppression; $P<0·01$ OVA fed versus saline fed).

The remaining mice were held for 7 days after the OVA feed (i.e. 14 days after immunization) and then killed to serve as spleen cell donors. Spleen cells, 10^8, were transferred intraperitoneally into each recipient mouse.

Recipient mice of Groups A (OVA-immunized saline-fed cells) and B (OVA immunized OVA-fed cells) were immunized with OVA in CFA approximately 4 hr after spleen cell transfer. They were tested for DTH 3 weeks later and results are shown in Fig. 3a. Spleen cells from immunized and OVA-fed donor mice suppressed the induction of a DTH response in naive recipients (64% suppression; $P<0·01$ group A versus group B).

The same donor cell preparations were also transferred into mice which had been immunized with OVA in CFA 1 week previously, i.e. at the time when a feed of OVA can suppress DTH. Group C received cells from OVA-immunized saline-fed donors and Group D cells from OVA-immunized mice. Assessment of the DTH responses was performed at 21 days after immunization. In this case, transfer of cells from OVA fed donors into sensitized mice had no effect on the subsequent DTH response ($P>0·05$; Group C versus Group D). Thus, the spleen cell suspension used for adoptive transfer did contain a

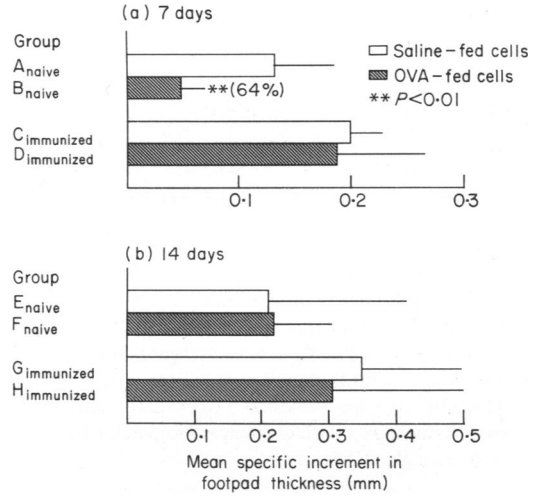

Figure 3. Cell transfer of DTH suppression from immunized and fed hosts. (a) Seven days after OVA feeding, 10^8 spleen cells from OVA-immunized OVA-fed donors were given either to naive (Group B) or Immunized (Group D) recipients and DTH responses were assessed as indicated in the text. Control groups (naive recipients, Group A; immunized recipients, Group C) received cells from OVA-immunized saline-fed donors. (b) Fourteen days after OVA feeding, 10^8 spleen cells from OVA-immunized OVA-fed donors were given either to naive (Group F) or immunized (Group H) recipients, and DTH responses were assessed as indicated in the text. Control groups (naive recipient, Group E; immunized recipient, Group H) received cells from OVA-immunized saline fed donors. DTH responses are expressed as mean specific increment in footpad thickness ± 1 SD deviation for groups of six to eight BDF1 mice.

population of suppressor cells, but these were active only when given to naive recipients before immunization, and were not able to suppress the expression of DTH.

14 days after OVA feed

The same protocol was used to assess whether cells capable of suppressing DTH expression were present in the spleen 14 days after OVA feeding (i.e. 21 days after immunization). As before, small groups of donor mice were retained and skin tested to confirm suppression of their DTH response to OVA (50% suppression; $P < 0{\cdot}01$ OVA fed versus saline fed).

Naive recipient mice of Groups E and F were given cells from OVA-immunized saline-fed donors and OVA-immunized OVA-fed donors, respectively, before immunization. Recipient Groups G (OVA-immunized saline-fed cells) and H (OVA-immunized OVA-fed cells) had been immunized 1 week before cell transfer. DTH responses were assessed 3 weeks after immunization, and the results are shown in Fig. 3b.

The spleen cell suspension from OVA-immunized OVA-fed mice was unable to suppress either the induction ($P > 0{\cdot}05$; Group E versus Group F) or the expression of the DTH response ($P > 0{\cdot}05$; Group E versus Group H) after transfer into recipient mice. Thus, the results suggest that the suppressor cells found in the spleen 7 days after feeding are no longer present at 14 days. Furthermore, the mechanism of post-immunization suppression by antigen feeding does not involve the OVA-specific suppressor cells which have been identified in previous models of oral tolerance.

DISCUSSION

The results presented here demonstrate that a single feed of 25 mg OVA given after immunization prevented subsequent DTH responses to the antigen, but had no significant effect on serum antibody responses. Furthermore, these experiments have failed to reveal a role for orally induced suppressor cells in this form of tolerance. Our study extends previous work from this laboratory by demonstrating that oral administration of antigen can influence profoundly not only the induction of a DTH response but also its systemic expression.

There are several previous reports that tolerance (or partial tolerance) of antibody responses can occur when antigen is fed after immunization (Lafont *et al.*, 1982; Bloch *et al.*, 1983; Saklayen *et al*, 1984). It is clear from our studies that expression of CMI may also be suppressed under these circumstances. Nevertheless, major differences between the two limbs of tolerance exist, in that multiple feeds are required to suppress the antibody response whereas a single feed is sufficient for suppression of DTH. The reason for these differences is not known but may reflect the different mechanisms of suppression involved. It is interesting to note that separate mechanisms of tolerance induction for antibody and DTH responses have also been proposed to operate when antigen is fed before immunization (Mowat *et al.*, 1982, 1986).

DTH suppression became less pronounced as the time interval between immunization and feeding increased (Fig. 1). It would be of interest, therefore, to determine whether, at some time after immunization, the DTH response no longer proved susceptible to orally induce suppression. This point would have direct bearing on the potential uses of the oral route for desensitization therapy aimed specifically at DTH responses. Experiments designed to answer this question are currently in progress. Secondly, of the two strains used in these experiments, a greater degree of suppression of the DTH response was consistently observed with BDF1 mice, compared to BALB/c mice (Fig. 1; Table 2; M. G. Bruce, unpublished observations). Strain differences in oral tolerance induction for antibody responses have been noted previously both when antigen is fed before immunization (Stokes, Swarbrick & Soothill, 1983) and after immunization (Lafont *et al.*, 1982). Similarly, Tomasi *et al.* (1983) have described differences between strains in the cell-mediated limb of oral tolerance as measured by T-cell proliferation *in vitro*. Our results suggest that these differences may also occur in the DTH response, although a wider range of mouse strains should be used to examine this. Experiments of this sort will help to define the basis for genetically determined differences in oral tolerance induction.

In view of the role of Ts cells in the suppression of DTH when antigen is fed before immunization (Mowat, 1985, 1986), experiments to investigate the relevance of these cells as a mechanism of post-immunization suppression were performed. Two different approaches were used, pharmacological inhibition of Ts cells, and adoptive transfer experiments.

Administration of 2dGuo after feeding and before immunization can abrogate the induction of oral tolerance by preventing the generation of antigen-specific Ts cells (Mowat, 1986). This agent, however, failed to influence suppression of DTH when immunization occurred before OVA feeding (Fig. 3). Nevertheless, it was possible that a population of 2dGuo-resistant suppressor cells were active in causing suppression. In

order to examine this, we transferred spleen cells from tolerant donor animals to naive recipients, before immunization and to recipients which had been immunized 1 week previously. A population of suppressor cells was present in the spleen at 7 but not 14 days after feeding. Since they prevented the induction phase of the DTH response, these cells resemble the 'classical' orally induced Ts cells described by others (Miller & Hanson, 1979; Mowat, 1985). However, the same spleen cells which transferred tolerance to naive mice did not suppress immune responses in sensitized animals (Fig. 3). Overall, therefore, the results suggest that tolerance for DTH in this model is not attributable to Ts cells.

Other mechanisms which could prevent the expression of DTH responses after challenge must be considered. One possibility is that feeding antigen after immunization induces a state of functional anergy in Th/DTH cells in the absence of Ts cells. This anergic state may manifest itself in an inability of the cell to respond to the stimuli normally associated with lymphocyte activation and proliferation. *In vitro*, anergy has been demonstrated readily in T-cell clones recognizing the HA antigen of influenza virus (Lamb & Feldman, 1984). Alternatively, phenotypic anergy for DTH may result from an inability of effector cells to migrate to the distant site of antigen challenge. A further possiblity is that the clonal expansion of Th/DTH cells is restricted by antigen feeding after immunization. The relevance of a constraint on expansion to the state of tolerance is uncertain, since DTH responses in our model have reached near maximal levels by Day 14 after immunization, yet a single feed of OVA at this time can still result in suppression of DTH (Fig. 1, Group f).

The reduction of the DTH response in immunized hosts following ingestion of antigen may have important clinical implications. For instance, if food-sensitive enteropathic disorders arise as a consequence of a breakdown in oral tolerance for DTH responses (Mowat & Ferguson, 1981), the induction (or re-establishment) of tolerance would be of great benefit to the individual. There are clinical reports of successful oral desensitization (Bierme *et al.*, 1979; Sullivan, Wedner & Parker, 1980).

In summary, we have shown that DTH responses can be suppressed by a single feed of 25 mg OVA given either 7 days before, on the same day or up to 14 days after immunization. We have been unable to demonstrate a role for suppressor cells in this form of tolerance, and thus it appears quite distinct from models of DTH oral tolerance studied previously (Miller & Hanson, 1979; Mowat *et al.*, 1982; Mowat, 1985, 1986). Our results support the concept that several mechanisms of immune regulation can be activated by feeding antigen. Furthermore, we propose that whichever mechanism is prominent depends not only on the immune response under study, but also the immune status (naive versus sensitized) of the host.

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