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The kinetics of oral hyposensitization to a protein antigen are determined by immune status and the timing, dose and frequency of antigen administration

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SUMMARY

We have investigated the immunological consequences of feeding a protein antigen to previously immunized animals. BALB/c mice were systemically primed with ovalbumin (OVA) in complete Freund's adjuvant (CFA) and fed with high (10 mg/g body weight), medium (1 mg/g body weight) or low (1 μg/g body weight) doses of OVA once (Day 1, 7 or 14) or sequentially for 5 days (Days 1–5, 7–11, 14–18). The specific IgG antibody response was suppressed only by early feeds of high-dose OVA (Days 1–5). Medium-dose OVA fed on Day 14 or low-dose OVA fed at any stage after immunization enhanced the IgG antibody response. In contradistinction, systemic delayed-type hypersensitivity responses (DTH) were usually suppressed by early feeds of high or medium doses of OVA but never after feeding low-dose OVA. The results suggest that systemic DTH and IgG antibody responses to oral antigen are subject to different control mechanisms in previously primed animals. Such responses depend on the immune status of the animal and are controlled by antigen dose, time and frequency of feeding. The immunological effects observed are also demonstrable following adoptive transfer of spleen cells collected 14 days after multiple feeds of high-dose OVA to immunized mice. Our findings suggest that oral hyposensitization after systemic immunization is regulated by (suppressor) spleen cells which are activated by gut-processed antigen.

INTRODUCTION

The systemic immune hyporesponsiveness induced by feeding thymus-dependent antigens to naive animals is well documented and has been termed oral tolerance (Ngan & Kind, 1978; Richman *et al.*, 1978; Mattingly & Waksman, 1978; Hanson *et al.*, 1979a; Kagnoff, 1980; MacDonald, 1982; Mowat, 1987). By extrapolation, the suppression of ongoing systemic immune responses following oral antigen administration is clearly of potential therapeutic benefit in the hyposensitization of allergic individuals. Nevertheless, the clinical efficacy of oral hyposensitization remains controversial, having been claimed by some groups (Brown, Goldberg & Shearer, 1982; Björksten *et al.*, 1986) but questioned by others (Golbert, 1975; Reismann, 1981). The underlying principles of gastrointestinal immune regulation and their effects on the modulation of ongoing immune responses have not been addressed in these clinical studies.

Studies of the effects of antigen ingestion after systemic immunization of animals have also generated controversial data. One recent publication suggests that systemic DTH responses can be suppressed by feeding antigen after immunization (Lamont *et al.*, 1988). In the field of humoral immunity, some investigators have demonstrated enhancement of IgE antibody responses (Bazin & Platteau, 1976; Jarrett *et al.*, 1976), whereas other groups observed suppression after long-term administration of antigen (Lafont *et al.*, 1982; Saklayen *et al.*, 1984). Similarly, IgG antibody responses were reported to be enhanced (Hanson *et al.*, 1979b; Titus & Chiller, 1981), suppressed (Lafont *et al.*, 1982; Bloch *et al.*, 1984) or unchanged (Saklayen *et al.*, 1984; Lamont *et al.*, 1988). Such discrepancies may be attributable to the different experimental conditions used, and in this study we have attempted to evaluate the role in oral hyposensitization of various factors such as antigen dose, the time between immunization and feeding and the frequency of antigen administration. Furthermore, in adoptive transfer experiments using the spleen cells of immunized and fed animals we have sought evidence that the suppression of systemic immune responses is mediated by spleen cells.

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MATERIALS AND METHODS

Animals

Groups of 6–15 female BALB/c mice (6–8 weeks old) were used. They were purchased from Charles River (Margate, Kent) and were maintained on an ovalbumin (OVA)-free diet (Labsure, Manea, Cambridge).

Antigen

Ovalbumin (OVA; Grade V, Sigma, Poole, Dorset) was dissolved in 0·15 M sterile saline and prepared at three different

concentrations: (i) high dose (10 mg/g body weight): 400 mg/ml (0·5 ml/feed); (ii) medium dose (1 mg/g body weight): 100 mg/ml (0·2 ml/feed); (iii) low dose (1 μg/g body weight): 100 μg/ml (0·2 ml/feed).

Systemic immunization

Mice were parenterally immunized in the left hind foot pad with 100 μg OVA emulsified in 0·05 ml complete Freund's adjuvant (CFA; Bacto H37Ra, Difco Labs, Surrey). The day of systemic immunization is subsequently referred to as Day 0, and the dates of oral administration are given relative to this systemic immunization.

Oral administration of antigen

Under halothane anaesthesia, mice were fed by gentle gavage using an 18-gauge feeding tube with rounded tip (Fine Science Tools Inc., North Vancouver, Canada). Mice in the experimental groups were fed with OVA, and those in the control groups were fed with saline only.

Blood samples

Animals were bled from the tail on Day 20 and the separated serum was stored frozen at −20° until required.

Measurement of serum IgG antibody responses

Anti-OVA IgG antibodies were assessed by an ELISA. Flat-bottomed microtitre plates (Linbro, Flow Labs Ltd, Rickmansworth, Herts) were coated with 100 μg/ml OVA dissolved in 0·05 M carbonate buffer, pH 9·6 (100μl/well), overnight at 4°. Free sites on the plates were blocked with 1% goat serum (ICH ImmunoBiologicals, Lisle, IL) in 0·15 M saline, containing 0·1% thiomersal (Sigma) and 0·05% Tween 20 (Sigma) for 1 hr at room temperature. After six washes with saline-Tween, serum samples (1/1000 dilution) and standards (0–2000 ng/ml affinity-purified mouse anti-OVA IgG) diluted in saline-Tween with 1% goat serum were incubated in duplicate for 1·5 hr at 37°. After another six washes, 100 μl of a 1/5000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Fc-specific, Jackson Immunoresearch Labs, West Grove, PA) were added and the plates incubated for 1·5 hr at 37°. After the final six washes, 100 μl of *p*-nitro-phenyl-phosphate (1mg/ml; Sigma) dissolved in 0·05M carbonate buffer containing 0·0001M $MgCl_2$, were added and the plates incubated at 37°. Absorbance at 405 nm was read by a Titertek Multiscan ELISA Reader (Flow Labs) and unknowns were interpolated using a logistic curve fitting programme from Titersoft (Flow Labs).

Measurement of systemic DTH responses

Three weeks after systemic immunization, the systemic DTH responses were determined by a footpad swelling test. Mice were injected into the right hind foot pad with 100 μg heat-aggregated OVA in 0·05 ml sterile saline. The footpad thickness was measured using a dial gauge microcaliper (Mitutoyo, MFG Co., Tokyo, Japan) both before and 24 hr after challenge. The differences between these two measurements were used in the group comparisons.

Adoptive transfer of spleen cells

After systemic immunization, donor animals (15 mice per group) were fed with high-dose OVA or saline for 5 days (Days 1–5). Two weeks after OVA feeding (i.e. 19 days after immunization), spleens were removed and placed into RPMI-1640 (Gibco, Paisley, Renfrewshire). Spleen cells were liberated from the splenic capsule using RPMI-1640 in a 1-ml syringe with a 25-gauge needle. Thereafter, the spleen cells were washed three times and injected intraperitoneally into naive recipients (10^8). The animals were immunized with 100 μg OVA in CFA within 24 hr.

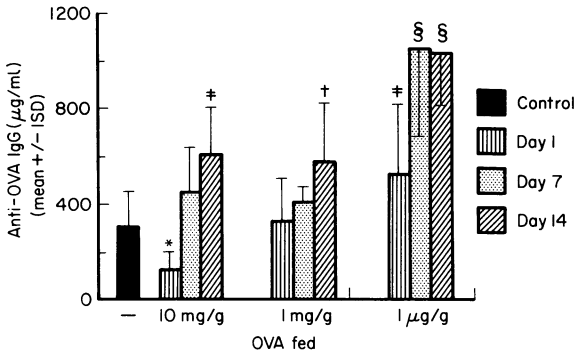

Figure 1. Serum anti-OVA IgG antibody responses after a single feed of OVA to previously immunized mice ($n = 6$). All mice were immunized on Day 0 and then fed with high (10 mg/g body weight), medium (1 mg/g body weight) or low (1 μg/g body weight) doses of OVA on Day 1, 7 or 14. Specific IgG antibodies were assessed on Day 20 and are expressed as the mean concentration for each group. The error bars represent 1 SD. Student *t*-test comparisons between various treatment groups and the control group (receiving saline alone): $*P < 0{\cdot}1$; $\dagger P < 0{\cdot}05$; $\ddagger P < 0{\cdot}02$; $\S P < 0{\cdot}001$.

Statistics

Both DTH and IgG antibody responses were expressed as means + 1 SD and all group comparisons were made using unpaired Student's *t*-tests.

RESULTS

Immunological effects of a single feed of OVA in systemically immunized animals

After systemic immunization, groups of experimental mice were subsequently fed with high (10 mg/g), medium (1 mg/g) or low (1 μg/g) doses of OVA on Days 1, 7 or 14; the control mice were fed with saline only. All mice were assessed for the production of anti-OVA IgG antibody and for systemic DTH responses 3 weeks after immunization. The results obtained are summarized in Figs 1 and 2.

High-dose feeds. When high doses of OVA were employed, the IgG antibody response observed was closely correlated with the time between immunization and antigen ingestion (Fig. 1). Although the IgG antibody response was enhanced by a high dose feed on Day 14 ($P < 0{\cdot}02$) the response after a feed on Day 1 was lower than that of the saline-fed control group, but the difference was not significant ($P > 0{\cdot}05$). However, in view of the significant suppression observed using multiple feeds of high dose OVA over Days 1–5 (Fig. 3), we repeated the Day 1 single challenge experiment using further groups of mice experimental group ($n = 6$) and controls ($n = 10$) and on this occasion the

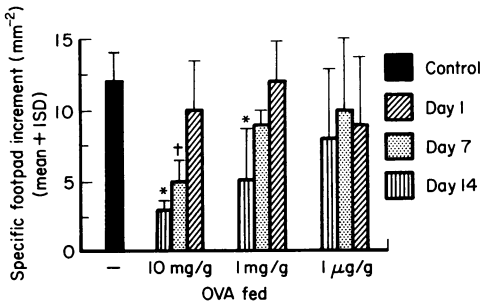

Figure 2. Systemic DTH responses after a single feed of OVA to previously immunized mice ($n=6$). All groups were immunized on Day 0 and then fed with high, medium or low doses of OVA on Day 1, 7 or 14, as in Fig. 1. DTH was measured 3 weeks after immunization, and is expressed as the mean increment in footpad thickness. The error bars represent 1 SD. Significant suppression of the DTH response relative to the control group: $*P<0{\cdot}01$; $\dagger P<0{\cdot}001$.

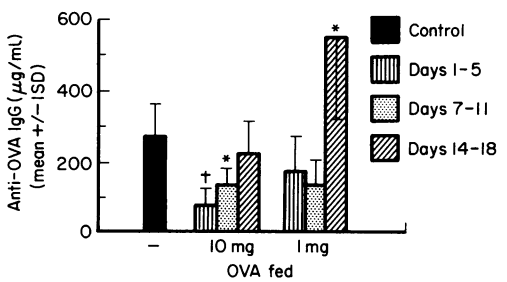

Figure 3. Serum anti-OVA IgG antibody responses after multiple feeds of OVA to previously immunized mice ($n=10$ for saline-fed group, $n=6$ for OVA-fed group). All mice were immunized on Day 0 and then fed with high or medium doses of OVA daily from Day 1 to 5, 7 to 11 or 14 to 18. Specific IgG antibodies were assessed on Day 20 and are expressed as the mean concentration for each group. The error bars represent 1 SD. Significant suppression or enhancement of specific antibody levels relative to the control group: $*P<0{\cdot}01$; $\dagger P<0{\cdot}001$.

antibody response of the OVA-fed group was significantly lower than that of the control group ($P<0{\cdot}02$; data not shown).

In contrast to the antibody responses, systemic DTH responses were profoundly inhibited by feeding antigen on Days 1 or 7 ($P<0{\cdot}001$) but not on Day 14 ($P>0{\cdot}1$; Fig. 2).

Medium-dose feeds. When a medium dose (1 mg/g body weight) of OVA was given, IgG antibody levels increased progressively with the time interval between feeding and immunization (Fig. 1), and the response was significantly enhanced by a feed on Day 14 ($P<0{\cdot}05$). By contrast, systemic DTH responses were preferentially suppressed by a feed on Day 1 ($P<0{\cdot}02$), but not on Day 7 ($P>0{\cdot}05$) or 14 (Fig. 2).

Low-dose feeds. When the low dose of OVA was used, there was no suppression in DTH responses (Fig. 2), but IgG antibody responses were enhanced by a feed on Day 1 ($P<0{\cdot}05$) and significantly boosted by feeds on Day 7 or 14 ($P<0{\cdot}001$; Fig. 1).

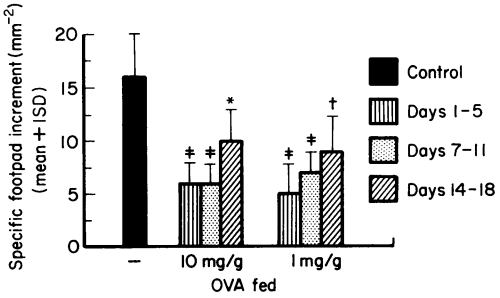

Figure 4. Systemic DTH responses after multiple feeds of OVA to previously immunized mice, as detailed in Fig. 3. DTH was measured 3 weeks after immunization, and is expressed as the mean increment in footpad thickness. The error bars represent 1 SD. Significant suppression of the DTH response relative to the control group: $*P<0{\cdot}02$; $\dagger P<0{\cdot}005$; $\ddagger P<0{\cdot}001$.

Effects of multiple feeds of OVA to immunized animals

The same basic protocol was used to study the effects of multiple feeds of high- or medium-dose OVA from Days 1–5, 7–11 or 14–18. The results are shown in Figs 3 and 4.

High-dose feeds. IgG antibody responses were severely suppressed by high-dose feeds from Days 1–5 ($P<0{\cdot}001$) or Days 7–11 ($P<0{\cdot}01$). However, this suppression was not obtained with feeds from Days 14–18 ($P>0{\cdot}3$; Fig. 3). In contrast, systemic DTH responses were profoundly inhibited by feeds from Days 1–5 or 7–11 (both $P<0{\cdot}001$) and from 14–18 ($P<0{\cdot}02$; Fig. 4).

Medium-dose feeds. The independence of IgG antibody and DTH responses was conspicuous when multiple feeds of medium-dose OVA were administered. Serum IgG antibody responses were unaffected by feeds from Days 1–5 but highly augmented by feeds from Days 14–18 ($P<0{\cdot}01$; Fig. 3). On the other hand, DTH responses were significantly suppressed by feeds from Days 1–5 ($P<0{\cdot}001$) or 14–18 ($P<0{\cdot}005$; Fig. 4).

Adoptive transfer of spleen cells after oral hyposensitization

The above experiments showed that both IgG antibody and DTH responses were best suppressed by repeated feeds of high-dose OVA as early as possible after immunization. Thus, in this study, systemically immunized donor mice were subsequently fed with saline or high-dose OVA from Days 1–5 (15 mice per group). Two weeks later (i.e. 19 days after immunization), the spleen cells of each group were pooled and injected intraperitoneally into naive recipients (10^8 cells per animal). Within 24 hr, the recipients and controls were systemically immunized with OVA in complete Freund's adjuvant. Thereafter, their IgG antibody responses were monitored on Days 9, 14 or 20, and DTH responses were measured on Day 21.

Antibody responses. The *donor* mice were bled immediately before splenectomy and serum IgG anti-OVA antibody levels measured. It was found that the anti-OVA IgG antibody responses of these animals were reduced by early feeds of high-dose OVA following immunization when compared to a saline-fed group (mean + 1 SD of 333 + 173 μg/ml in the saline-fed group and 82 + 54 μg/ml in the OVA-fed group; $P<0{\cdot}001$).

Compared with a control group which received no spleen cells, both of the *recipient* groups showed a significantly raised

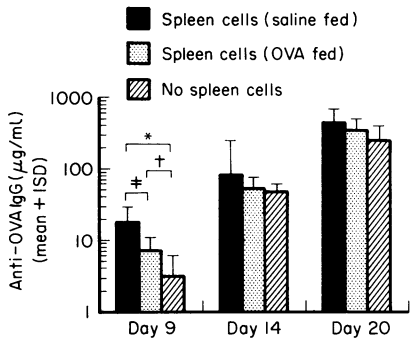

Figure 5. IgG antibody responses in mice after transfer of 10^8 spleen cells collected following systemic immunization and oral hyposensitization. Donor mice ($n = 15$) were immunized with ovalbumin on Day 0 and then fed with saline or OVA (10 mg/g body weight) from Day 1 to 5. Fourteen days after the last feed spleen cells were transferred into naive mice. The recipients ($n = 6$) were immunized immediately with OVA in complete Freund's adjuvant. Serum anti-OVA IgG antibodies were assessed 9, 14 and 20 days after immunization and are expressed as the mean concentration for each group. The error bars represent 1 SD. Statistically significant differences between groups on Day 9 are indicated: *$P < 0{\cdot}001$; †$P < 0{\cdot}02$; ‡$P < 0{\cdot}01$.

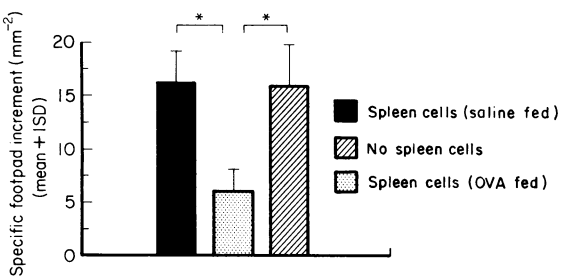

Figure 6. Spleen cell transfer of DTH suppression after oral hyposensitization. Experimental conditions as described in legend to Fig. 5. DTH responses were measured 3 weeks after immunization and are expressed as the mean specific increment in footpad thickness. The error bars represent 1 SD. The DTH response of the group receiving spleen cells from OVA donors was significantly lower than the response of both the group receiving spleen cells from saline-fed donors and a control group which received no spleen cells: *$P < 0{\cdot}005$.

IgG response 9 days after immunization (spleen cells with saline feed $P < 0{\cdot}001$, spleen cells with OVA feed $P < 0{\cdot}02$; Fig. 5). Those mice receiving spleen cells after oral hyposensitization had a significantly lower IgG antibody response on Day 9 than mice receiving spleen cells from donors fed with saline ($P < 0{\cdot}01$). Similar patterns of response were observed on both Day 14 and Day 20, but the differences were not statistically significant.

DTH responses. The same mice showed a profound suppression in DTH response on Day 21 ($P < 0{\cdot}005$; Fig. 6). As with previous experiments, it was observed that the suppression in DTH responses during oral hyposensitization was more pronounced and more prolonged than the suppression of IgG antibody responses (Figs 5 and 6).

DISCUSSION

The results presented here indicate that oral hyposensitization (oral tolerance) and oral immunization (priming) can be induced in previously sensitized mice by feeding OVA in a dose-dependent fashion. Systemic DTH responses were preferentially suppressed by feeds of high-dose (10 mg/g body weight) or medium-dose (1 mg/g body weight) OVA. IgG antibody responses were only suppressed by multiple feeds of high-dose OVA close to the time of systemic immunization. In contrast, enhanced IgG antibody responses were readily induced by feeds of low-dose OVA (1μg/g body weight) at any stage after systemic immunization.

Suppression of systemic immune responses in naive animals after a single feed of a protein antigen has been demonstrated by several groups, most of whom have used T-dependent antigens (Ngan & Kind, 1978; Mattingly & Waksman, 1978; Hanson *et al.*, 1979b; Strobel *et al.*, 1983; Mowat, 1987). However, attempts to suppress an established immune response by subsequent oral administration of antigen have been largely unsuccessful (Hanson *et al.*, 1979b; Titus & Chiller, 1981). A few previous reports have suggested that (partial) suppression of antibody responses can be achieved if the antigen is fed for prolonged periods after immunization. Such studies have been performed both in experimental animals (Lafont *et al.*, 1982; Bloch *et al.*, 1984; Saklayen *et al.*, 1984) and in man (Wortmann, 1977; Bierme *et al.*, 1979; Brown, Goldberg & Shearer, 1982; Björksten *et al.*, 1986).

Our studies and those of Lamont *et al.* (1988) clearly indicate that there is also a suppressive effect on cell-mediated immunity. Suppression of both limbs of the immune system has generally been observed when the antigen has been fed before immunization (Mowat *et al.*, 1982; Strobel *et al.*, 1983; Strobel & Ferguson 1984, 1987; Mowat, 1987). Published work suggests that the cellular and humoral limbs of the immune system are under different control (Chiller & Weigle, 1972; Strobel & Ferguson, 1987; Lamont *et al.*, 1988). Delayed-type hypersensitivity responses are suppressed at lower antigen dose levels than IgG antibody responses (Strobel *et al.*, 1983; Mowat, 1987) and such suppression is maintained for over 18 months (Strobel & Ferguson, 1987).

The basic mechanisms underlying oral tolerance remain poorly understood and are apparently different for soluble protein antigens (Richman *et al.*, 1978; Ngan & Kind, 1978; Hanson *et al.*, 1979b) and particulate antigens (Mattingly & Waksman, 1978; Kagnoff, 1980; MacDonald, 1982) in which tolerance can be preceded by a period of systemic priming (David, 1979). In general, only T-dependent antigens can induce T-cell tolerance for both limbs of the immune response and B cells remain potentially active (MacDonald, 1982). Active T-cell suppression *in vivo* has been demonstrated by oral administration of protein antigens (Richman *et al.*, 1978; Ngan & Kind, 1978; Hanson *et al.*, 1979b) and particulate antigens (Mattingly & Waksman, 1978; Kagnoff, 1980; MacDonald, 1982). Adoptive spleen cell transfer studies from tolerized animals into naive recipients were performed to examine the role of active (possibly T-suppressor cell mediated) suppression in our system. The results suggest that only delayed-type hypersensitivity responses are suppressed for a prolonged period after the transfer of cells, whereas IgG responses are only temporarily suppressed during the induction phase (Day 9) and later escape immunological

suppression with the result that IgG antibody levels are indistinguishable from control responses. A recent report (Lamont *et al.*, 1988) suggests that the transfer of DTH suppression by spleen cells could have been mediated by 2-deoxyguanosine-resistant T-suppressor cells which might only be active during the induction phase in naive animals, since the injection of spleen cells after immunization did not suppress delayed-type hypersensitivity responses in their system. Further experiments to define the temporal relationship and the cell-type responsible for this suppression are clearly needed.

Other mechanisms may also be active in preventing DTH expression in these animals, and the decline in the capacity to tolerize animals in our experiments seems to correlate with increasing antibody production. These results are in agreement with another report in which oral tolerance to OVA was partially inhibited by prior injection of passive antibodies (Hanson *et al.*, 1979a). However, our observations differ in one important aspect. In the studies presented here, animals were actively sensitized and then suppressed during the induction phase of the immune response and were not passively sensitized. This discrepancy makes it highly unlikely that antigen availability or the priming effects of antigen–antibody complexes could explain our results.

It is also conceivable that feeding of antigen after immunization induces a (temporary) functional anergic state in T-helper cells responsible for the expression of delayed-type hypersensitivity in the absence of T-suppressor cells. However, the antibody suppression observed in animals fed high doses of OVA would argue against this possibility. The observed priming effect for IgG antibody responses after feeding low doses of antigen to previously sensitized animals is compatible with earlier studies in which secondary IgE responses were evoked by feeding low doses of ovalbumin to previously immunized rats (Bazin & Platteau, 1976; Jarrett *et al.*, 1976).

The reduction of delayed-type hypersensitivity responses and antibody responses in the immunized host by oral feeding has important clinical implications in the treatment of food protein sensitive enteropathies. These enteropathies may represent a breakdown of oral tolerance. Reinduction of this state of tolerance through antigen administration would be of great clinical benefit and has already been attempted for some selected non-food allergens (Wortmann, 1977; Bierme *et al.*, 1979; Brown *et al.*, 1982; Björksten *et al.*, 1986). A better understanding of the underlying mechanisms and the possible effects of immunoregulatory gut-processed peptides could open up new therapeutic avenues and provide novel approaches to the generation of oral vaccines.

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