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# The immunological consequences of feeding cholera toxin

## II. MECHANISMS RESPONSIBLE FOR THE INDUCTION OF ORAL TOLERANCE FOR DTH

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### SUMMARY

The mechanisms behind the induction of oral tolerance after feeding cholera toxin (CT) were examined using cell and serum transfer protocols. The feeding of CT or cholera toxoid (TD) induced a splenic cell capable of inhibiting the induction of systemic delayed-type hypersensitivity (DTH) but not humoral immunity. Depletion studies showed that this cell was Thy-1.2 positive. Transfer experiments suggested that suppressor cell activity was present in the mesenteric lymph nodes (MLN) and spleens of donor mice 1 week but not 3 days after feeding CT. When spleen cells were transferred to syngeneic recipients at various times after immunization, they were more effective at inhibiting systemic DTH when transferred within a short time of immunization. If the cells were transferred 6 days after immunization they no longer suppressed the development of DTH, which suggested that they inhibit the afferent limb of this immune response. This has been confirmed by the failure of a tolerogenic dose of CT, administered by gavage, to suppress the activity of mature effector TDTH cells. Serum collected 1 hr after feeding CT also suppressed the induction of systemic DTH. However, the tolerogenic activity of CT-fed serum was abrogated by the pretreatment of recipients with cyclophosphamide (Cy) (100 mg/kg), suggesting that this activity is mediated through the induction of suppressor cells. Transfer of fed serum, however, did not induce the splenic suppressor cell described above and we would suggest that several mechanisms may operate in the mucosal regulation of systemic DTH.


### INTRODUCTION

Protein antigens may induce a range of immune responses when fed, but most commonly generate a state of oral tolerance (Mowat, 1987). Oral tolerance has been shown to be the result of a number of regulatory mechanisms. These mechanisms, which are not always operative in every case, include antigen-processing (Bruce & Ferguson, 1986), antibodies (Kagnoff, 1978), antigen–antibody complexes (André *et al.*, 1975), soluble suppressor factors (Mattingly, Kaplan & Janeway, 1980), and both T- and B-suppressor cells (Mowat, 1987). The control of systemic antibody has been most frequently studied, but studies which have looked at the control of systemic DTH after the oral administration of protein antigens have shown that two mechanisms predominate. Suppressor T cells and antigen-processing by the gastrointestinal tract have both been implicated in the mucosal regulation of systemic DTH (Miller & Hanson, 1979; Strobel *et al.*, 1983; Bruce & Ferguson, 1986; Mowat, 1986).

Cholera toxin (CT) is not a typical protein antigen. It has both lectin-like qualities which allow it to adhere to the surface of enterocytes and lymphocytes alike, and enzymatic actions which enable it to activate irreversibly adenylate cyclase within all eukaryotic cells (Van Heyningen, 1983). Both these actions have been reported to modulate immune responses (McKenzie & Halsey, 1984; Lycke & Holmgren, 1986) and it has been shown that feeding CT induces both systemic and local antibody production (Elson & Ealding, 1984a). Despite CT's capacity to induce systemic DTH after parenteral administration even without the use of adjuvant (Kay, 1987; Kay & Ferguson, 1989a), its ability to induce helper T cells for systemic antibody after feeding (Elson & Ealding, 1984b) and its facility for abrogating the induction of oral tolerance to unrelated antigens after co-feeding (Elson & Ealding, 1984a), CT inhibits the induction of specific systemic DTH after oral administration (Kay & Ferguson, 1989b). We have examined the mechanisms by which CT induces oral tolerance for systemic DTH, and in particular, we have studied its capacity to stimulate specific DTH suppressor cells and tolerogenic post-randial serum fragments as have been described after feeding ovalbumin (Miller & Hanson, 1979; Strobel *et al.*, 1983; Bruce & Ferguson, 1986).

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## MATERIALS AND METHODS

*Animals*

Both male and female adult BALB/c mice between the ages of 6–10 weeks were used. Animals were bred and maintained in the Animal Unit, Western General Hospital, Edinburgh.

*Antigens*

Purified cholera toxin (CT; Choleragen, List Biological Labs. Inc., Campbell, CA), five times recrystallized ovalbumin (OVA, Sigma Chemical Co., Poole, Dorset) and immunopurified formalinized cholera toxoid, which was the kind gift of Dr R. O. Thompson, Wellcome Labs., Beckenham, Kent, were all used in this study.

*Immunization and assessment of systemic immunity*

Animals were immunized with 1 $\mu$g CT in complete Freund's adjuvant into the hind footpad on Day 0. Two weeks later (Day 14) the animals were challenged intradermally into the contralateral hind footpad with 5 $\mu$g TD dissolved in saline. The increment in footpad swelling was measured in mm after 24 hr. Eight days after this (Day 22), the animals were bled out and their serum assayed for IgG and IgA antitoxin antibody levels as described in the accompanying paper (p. 410).

*Oral immunization*

Animals were fed either 1 $\mu$g CT or 5 $\mu$g TD in ABS (PBS pH 7·6 with 6% $NaHCO_3$) or ABS alone. Gavage was performed using a rigid steel feeding tube with a rounded end under light ether anaesthesia.

*Depletion of Thy-1.2-positive spleen cells*

Spleens from donor mice were obtained 1 week after feeding. The spleens were cut up, passed through a fine gauge wire mesh and made into a single cell suspension in RPMI-1640 (Flow Laboratories Ltd, Irvine, Ayrshire). A total of $2 \times 10^7$ spleen cells/ml were incubated for 30 min at room temperature with a 1:1000 dilution of monoclonal IgM anti-Thy-1.2 antibody (F7D5; Serotec Ltd, Kidlington, Oxford). The cells were washed three times in medium and then incubated for 40 min at 37° with a 1:10 dilution of rabbit serum that had been previously absorbed with normal mouse spleen cells for 1 hr at 4°. The viability of the cells which remained was always greater than 95%.

*Adoptive transfer of DTH effector cells*

Draining popliteal and inguinal lymphoid cells were obtained from mice immunized 2 weeks earlier with 1 $\mu$g CT in CFA and made into a single cell suspension in RPMI-1640. A total of $10^7$ lymphoid cells were injected intradermally into the hind footpad of syngeneic recipients in the presence of 5 $\mu$g TD. Total volume of inoculate was 50 $\mu$l and DTH was assessed by the incremental change in footpad thickness measured 24 hr after cell transfer.

*Statistics*

The data shown below represents one example from experiments that were performed at least three times. The exception is the experiment illustrated in Fig. 6b, which was performed only once.

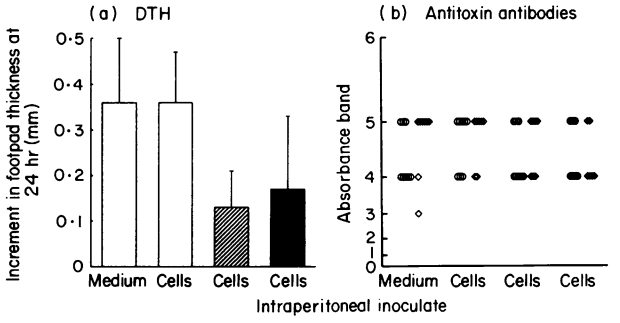


**Figure 1.** The capacity of orally primed splenocytes to transfer oral tolerance. Animals were given $10^8$ splenocytes obtained 1 week after feeding either ABS (□) CT (■) or TD (■) were transferred i.p. to syngeneic recipients on the same day as they were immunized i.d. with CT in CFA. Two weeks later the animals were footpad challenged with TD and 8 days after this were bled out for IgG and IgA antitoxin antibody estimation. The above graphs represent (a) the mean footpad responses measured 24 hr after footpad challenge $\pm$ 1 SD and (b) the individual IgG (○) and IgA (◇) antitoxin responses measured in isotype-specific ELISAs.

Means and standard errors of footpad thickness were compared using the Student's *t*-test and antitoxin antibody results were compared using the Wilcoxon rank sum test. Statistical significance was reached when $P < 0{\cdot}05$.

## RESULTS

**Effect of transfer of orally primed splenocytes on the induction of systemic immunity**

Spleen cells were obtained from mice fed either ABS, 1 $\mu$g CT or 5 $\mu$g TD 1 week earlier. One hundred million spleen cells (an amount representative of the contents of an average adult BALB/c mouse's spleen) were transferred intraperitoneally to naive syngeneic recipients on the same day as they were intradermally immunized with 1 $\mu$g CT in CFA (Day 0). Two weeks later these animals were footpad challenged with 5 $\mu$g TD and the increment in footpad thickness measured after 24 hr (Day 15). One week later (Day 22), these animals were bled and their IgG and IgA antitoxin antibody levels measured. The results of this experiment are shown in Fig. 1.

Mice which received $10^8$ spleen cells from ABS-fed donors had similar DTH and antitoxin antibody responses to sham-injected controls. Animals who received spleen cells from mice fed 1 $\mu$g CT or 5 $\mu$g TD 1 week earlier had significantly suppressed DTH responses ($P < 0{\cdot}01$ and $P < 0{\cdot}05$, respectively) but their humoral immune responses were no different from those observed in the other groups.

**The effect of depletion of Thy-1.2-positive cells on the ability of spleen cells to transfer oral tolerance**

Animals were given $10^8$ spleen cells from ABS- or CT-fed donors on the same day as they were immunized with CT in CFA. The CT-fed spleen cells were either untreated or treated with anti-Thy-1.2 monoclonal antibody or absorbed rabbit complement or a combination of the two. Animals given ABS-fed cells had a

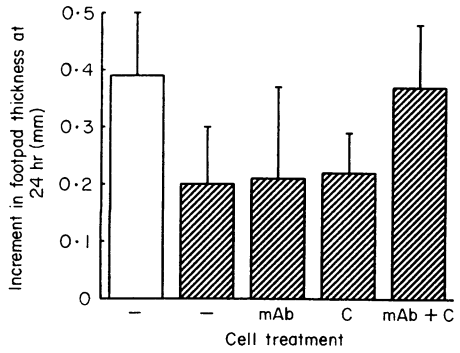


**Figure 2.** The effect of T-cell depletion on the transfer of oral tolerance. Cells from ABS- (□) or CT-fed (■) donors were transferred to recipients on the same day as they were immunized having been left untreated (–) or treated with either anti-Thy-1.2 (mAb), absorbed rabbit complement (C) or a combination of the two. The bars above represent the footpad responses of these recipients ± 1 SD 24 hr after footpad challenge.

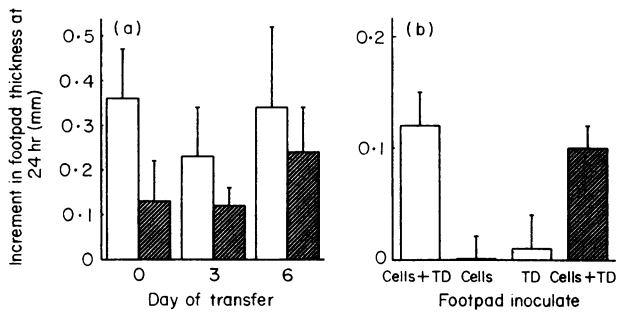


**Figure 3.** The mode of action of orally-induced tolerance. (a) Animals were given $10^8$ spleen cells from donors fed ABS (□) or CT (■) on the same day as or 3 or 6 days after they were immunized with CT in CFA. Two weeks later the recipients were challenged with TD and the above bars represent the increment in footpad thickness ± 1 SD 24 hr after footpad challenge. (b) The mean footpad responses ± 1 SD measured 24 hr after ABS- (□) or CT-fed (■) recipients were given either $10^7$ DTH effector cells, 5 μg TD or both, i.d.

normal DTH response (Fig. 2) and recipients of untreated CT-fed spleen cells had significantly suppressed responses ($P < 0{\cdot}01$).

Treating the CT-fed spleen cells with either anti-Thy-1.2 or complement alone did not affect their ability to transfer significant tolerance ($P < 0{\cdot}05$ and $P < 0{\cdot}01$, respectively). Depletion of the Thy-1.2-positive spleen cell population by anti-Thy-1.2 followed by complement, which accounted for approximately 40% of these cells, abrogated the ability of CT-fed cells to transfer suppression.

### The effect of the timing of cell transfer on the suppression of systemic DTH responses

Saline- and CT-fed spleen cells were transferred to syngeneic recipients on the same day as or 3 or 6 days after immunization. The results of this experiment are shown in Fig. 3a. Saline-primed cells did not suppress the recipients' DTH responses regardless of the time of transfer. CT-fed splenocytes significantly suppressed recipients' DTH responses when transferred either on the same day as ($P < 0{\cdot}001$) or 3 days after ($P < 0{\cdot}05$) immunization. However, CT-primed spleen cells did not significantly suppress the induction of systemic DTH when transferred 6 days after immunization. This experiment suggested that orally induced splenic suppressor cells inhibit the afferent limb of the systemic DTH response.

In order to confirm this, mature effector cells were transferred in the presence of TD to animals that had been fed either ABS or CT 1 week earlier. DTH effector cells were able to transfer significant DTH when transferred with TD into ABS-fed recipients (Fig. 3b) but neither caused a significant swelling response when transferred alone. Feeding animals CT failed to suppress the DTH response transferred by mature effector cells, which confirms that the tolerance observed is due to the inhibition of the afferent limb of this systemic response.

### The migration pattern of the orally induced suppressor cell

$10^8$ cells were transferred from the mesenteric lymph nodes (MLN) and spleens of saline- and CT-fed donors both 3 and 7 days after feeding. Recipient animals were immunized on the same day as cell transfer and footpad challenged, as before, 2 weeks later. The results of this experiment are shown in Fig. 4.

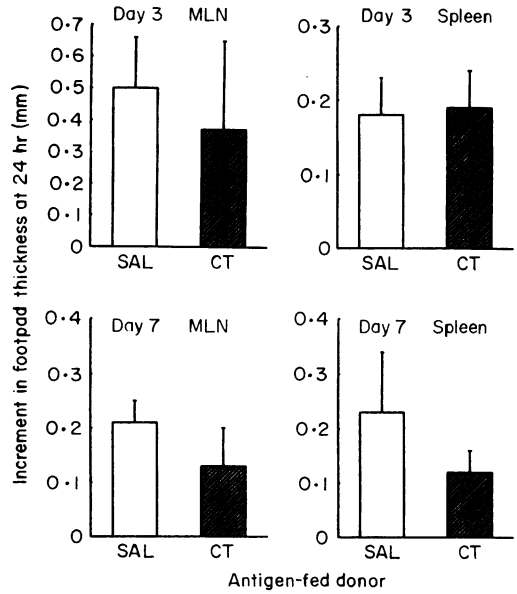


**Figure 4.** The migration of orally induced suppressor cells. $10^8$ MLN or spleen cells from ABS- (□) or CT-fed (■) donors were transferred either 3 or 7 days after feeding. Recipient animals were immunized on the day of transfer and footpad challenged 2 weeks later. The bars represent the mean increment in footpad thickness ± 1 SD 24 hr after footpad challenge.

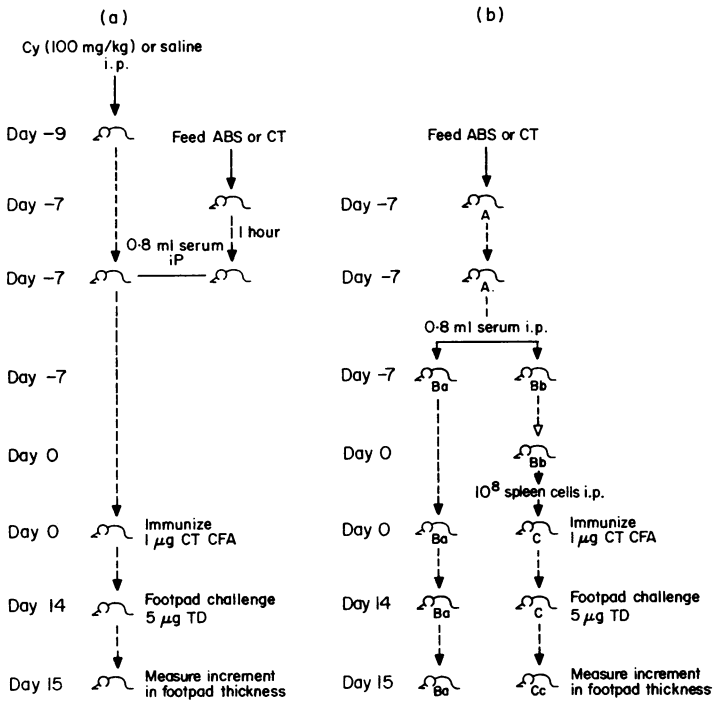


**Figure 5.** The experimental protocols for examining the tolerogenic effects of CT-fed serum. These protocols examine the ability of CT fed serum to (a) transfer oral tolerance to a Cy-pretreated recipient, and (b) induce a splenic suppressor cell 1 week after transfer.

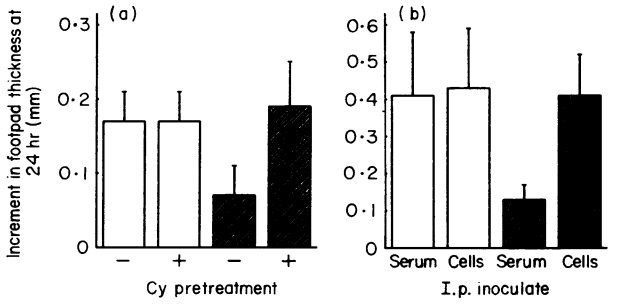


**Figure 6.** The effect of transfer of CT-fed serum on the infuction of systemic DTH. These experiments show the effect of transfer of CT- (■) and ABS-fed (□) serum (a) on the induction of systemic DTH in sham-injected and Cy-pretreated recipients and (b) on the induction of splenic suppressor cells. The protocols for these experiments are shown in Fig. 5 a and b, respectively. The bars above represent the mean increment in footpad thickness ± 1 SD 24 hr after footpad challenge.

CT-fed MLN and spleen cell recipients mounted similar DTH responses to ABS-fed cell controls when these tissues were obtained 3 days after feeding. However, either MLN or spleen cells obtained 1 week after feeding CT significantly inhibited the induction of systemic DTH compared with ABS-fed controls ($P < 0{\cdot}05$, in both cases).

### The suppressive effects of serum obtained 1 hr after feeding CT

Serum obtained 1 hr after feeding was tested for its capacity to suppress the induction of DTH. These experiments also examined the sensitivity of such suppression to cyclophosphamide pretreatment and the ability of fed serum to induce the suppressor cell described above. The protocols employed in these experiments are shown in Fig. 5.

In the first experiment (Fig. 5a), donor animals were fed with either ABS or CT and were bled out after 1 hr and the serum from each group of animals was pooled. Eight-hundred microlitres of serum (an amount considered to be representative of the circulating serum volume of an adult mouse) were transferred intraperitoneally to recipients. Recipient animals for both CT- and ABS-fed serum had been pretreated 2 days earlier (Day −9) with an intraperitoneal injection of cyclophosphamide (Cy; 100 mg/kg) or an equivalent volume of saline. Seven days after receiving the serum (Day 0), the recipient animals were immunized with CT in CFA and footpad challenged with TD 2 weeks later (Day 14). The DTH response was measured 24 hr after footpad challenge (Day 15). This experiment revealed (Fig. 6a) that ABS-fed serum recipients mounted normal DTH responses regardless of whether they had been pretreated with Cy or not. Recipients of CT-fed serum had suppressed DTH responses ($P < 0{\cdot}002$) but this suppression was reversed by pretreatment with Cy.

In the second experiment (Fig. 5b), donor animals (labelled A in the diagram) were fed with either ABS or CT, bled out after 1 hr and the serum from each group pooled and transferred to the next set of recipients (labelled Ba and Bb). Ba recipients were given the serum intraperitoneally and 7 days later immunized with CT as before. The second group of serum recipients, Bb, were intermediate hosts and 1 week after being given the serum were killed and their spleens removed. One-hundred million spleen cells from Bb donors were then transferred to the final set of recipient mice (labelled C) on the same day as they were immunized with CT in CFA. From that stage Ba and C recipients were treated identically as shown above. In this way, the quality of fed serum could be tested as well as its ability to induce splenic suppressor cells.

The results displayed in Fig. 6b show that splenic lympho-

cytes from ABS-fed serum recipients do not significantly alter the systemic DTH responses observed in animals receiving ABS-fed serum directly. As in the previous experiment, CT-fed serum was able to significantly suppress the induction of systemic DTH ($P<0{\cdot}001$). However, CT-fed serum did not induce suppressor cells in the spleens of recipient mice after 7 days, the DTH responses in these spleen cell recipients not being significantly different from those observed in ABS-fed serum recipient controls.

## DISCUSSION

Our previous work has demonstrated that feeding CT induces oral tolerance for systemic DTH but not antitoxin antibody responses (Kay & Ferguson, 1989b). This study extends these findings and demonstrates that at least two separate mechanisms are responsible for the suppression of systemic DTH—a Thy-1.2-positive suppressor cell found in the MLN and spleens of animals 1 week after feeding and a serum component present 1 hr after CT has been fed.

Suppressor T cells are one of the most common mechanisms found to be responsible for oral tolerance (Mowat, 1987). Cells which suppress systemic antibody have been found after feeding, initially in the Peyer's patch (PP) and subsequently in the MLN, thymus and spleen (Ngan & Kind, 1978; Mattingly & Waksman, 1978). A similar pattern of migration has been found for helper T cells induced by feeding CT (Elson & Ealding, 1984a). We have not examined the PP, but we have successfully demonstrated the presence of suppressor cells in both the MLN and spleen 1 week after CT was fed. Suppressor T cells which inhibit the induction of DTH have been found in the spleen 1 week after feeding OVA and these were shown to inhibit the afferent limb of the immune response (Miller & Hanson, 1979). Oral tolerance for CT as well as for OVA has been found to be Cy-sensitive (Mowat, 1986, 1987; Kay & Ferguson, 1989b) and it has been demonstrated that Cy preferentially inhibits the actions of cells suppressing the afferent limb of the immune response (Asherson *et al.*, 1980). It is therefore not surprising that we have found the orally induced suppressor T cell to inhibit the appearance of DTH but that feeding CT did not suppress the activity of mature DTH effector cells.

In this study, a formalinized but immunologically cross-reactive toxoid (TD) was fed as well as untreated CT in order to ascertain the role of CT's biochemical properties on the induction of oral tolerance. Formaldehyde has been shown to effect a reductive alkylation of lysine and tyrosine residues (Habeeb, 1969; Pancake & Nathenson, 1973) and has been shown to reduce the ability of certain antigens to provoke antibody production (Pancake & Nathenson, 1973; Pierce, 1978). It has been shown that TD is poorer than CT at priming for antibody but is equally immunogenic at boosting primed antibody responses and stimulating DTH (Pierce, 1978; Kay & Ferguson, 1989a, b). As B and suppressor T cells recognize conformational determinants (Feldmann & Kontiainen, 1976; Chesnut, Enders & Grey, 1980) and T-helper/DTH cells respond to short lengths of primary sequence presented in the context of class II MHC antigens (Chesnut *et al.*, 1980; Unanue *et al.*, 1984) it might be expected that both proteins would differ in their ability to induce oral tolerance. On the contrary, we have shown that both antigenic forms induce totally cross-reactive oral tolerance (Kay & Ferguson, 1989b) and that both induce suppressor cells after feeding. We would suggest that although orally primed suppressor T-cell determinants may be conformational they are not necessarily the same as those seen by B cells and recent studies have demonstrated that conformational H-2 determinants on target cells specifically recognized by cytotoxic T cells are left unaltered by formalinization despite the loss of the B-cell reactivity (Hua *et al.*, 1987).

Another mechanism responsible for the down-regulation of DTH after feeding is the 'intestinal processing' of antigen (Strobel *et al.*, 1983; Bruce & Ferguson, 1986; Bruce *et al.*, 1987). In this system, serum obtained 1 hr after feeding OVA to mice has been shown to be tolerogenic for DTH only. The changes found in fed OVA are not simply due to denaturation or deaggregation and intact intestinal lymphoid cells are necessary for its generation (Bruce & Ferguson, 1986; Bruce *et al.*, 1987). Although Cy pretreatment of donors does not affect the generation of this tolerogen, Cy has been shown to neutralize the tolerogenic effects of fed serum on recipient animals (Strobel *et al.*, 1983). This finding suggests that fed serum induces suppressor T cells and although we have been able to confirm the effect of Cy on the generation of tolerance in syngeneic recipients we have been unable to demonstrate the generation of splenic suppressor cells after intraperitoneal serum transfer.

Although we cannot exclude that the intraperitoneal transfer of serum alters either the migration of suppressor cells to the spleen or even their induction there, this is not the only model whereby DTH is down-regulated after feeding in the absence of demonstrable splenic suppressor cells. When denatured OVA is fed, for example, oral tolerance for DTH has been observed in the absence of splenic suppressor cells (Mowat, 1986) and established DTH responses to OVA can be suppressed by feeding, again in the absence of detectable splenic suppression (Lamont *et al.*, 1987). Furthermore, tolerogenic anti-T-cell antibodies capable of inhibiting the DTH responses to allogeneic spleen cells have also been described after the administration of antigen into the superior mesenteric vein (Sato *et al.*, 1988). Taken together, these results strongly suggest that several mechanisms are operative in the mucosal regulation of systemic DTH.

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