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The immunological consequences of feeding cholera toxin

I. FEEDING CHOLERA TOXIN SUPPRESSES THE INDUCTION OF SYSTEMIC DELAYED-TYPE HYPERSENSITIVITY BUT NOT HUMORAL IMMUNITY

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SUMMARY

Immunization of adult BALB/c mice with 1 μg cholera toxin (CT) in complete Freund's adjuvant (CFA) induced both humoral (IgG and IgA) and cell-mediated (DTH) immunity. Although an immunopurified, formalinized, cholera toxoid (TD) in CFA was inferior to the native holotoxin at inducing antitoxin antibodies, both cholera-derived antigens were equally immunogenic for specific DTH. When mice were fed either 1 μg CT or 5 μg TD 1 week before immunization, the induction of DTH was inhibited but the development of specific antibody was the same as in sham-fed controls. A feed of 10 μg CT not only suppressed the induction of DTH but also enhanced the IgG antitoxin responses measured 1 week after immunization. A dose of TD (50 μg), with a similar cholera toxin B subunit content, also induced oral tolerance for DTH but had no effect on the subsequent development of humoral immunity. The smallest doses of CT or TD fed (0·1 μg and 0·5 μg, respectively) failed to affect the development of either limb of the systemic immune response. These results suggest that oral tolerance for DTH is not consequent upon the metabolic actions of CT but that stimulation of systemic antibodies after enteric administration may be. Pretreating mice with cyclophosphamide (Cy) (100 mg/kg) before feeding CT abrogated the induction of oral tolerance for DTH but had no effect on humoral immunity, suggesting that suppressor T cells may be responsible for the induction of oral tolerance in these anaimals.

INTRODUCTION

CT is a protein molecule of molecular weight 80,000. It consists of a central A subunit (27,000 MW) surrounded by a pentameric ring of B subunits (11,000 MW each). The B subunits bind to GM1 gangliosides on the surface of all eukaryotic cells and facilitate the entry of a portion of the central toxic A subunit (A1 peptide). This peptide binds to the stimulatory regulatory component of adenylate cyclase leading to essentially irreversible activation of this enzyme (Van Heyningen, 1983). The subsequent accumulation of intracellular cAMP has differing effects according to the nature of the cell stimulated by the toxin. It is this course of events which is believed to be one of the factors which allows CT to modulate the immune responses both to itself and other unrelated antigens (Lindholm *et al.*, 1976; Elson & Ealding, 1984a; Lycke & Holmgren, 1986).

CT is a powerful immunogen and even in the absence of adjuvant is capable of evoking marked antitoxin antibody and B-cell responses in only microgram quantities (Pierce, 1978; Fuhrman & Cebra, 1981; Elson & Ealding, 1984b). Recently it has also been shown that CT may also induce systemic cell-mediated immunity (CMI) after intradermal immunization in adult BALB/c mice (Kay & Ferguson, 1989). Although a number of factors may affect the outcome, feeding protein antigens usually results in a state of oral tolerance both for systemic CMI and humoral immunity (Mowat, 1987). CT is atypical in this respect as repeated oral administration has been shown to stimulate both local and systemic antibody production (Elson & Ealding, 1984b; Lange, Lönnroth & Nygren, 1984). It is unclear whether this property is due to the biochemical actions of the toxin, alluded to above, or is a consequence of its inherent antigenic nature. The effect of feeding CT on the subsequent induction of DTH has never been examined.

The work described below attempts to address both these questions by examining both effector limbs of the systemic immune response in fed and unfed BALB/c mice. In addition, feeding a formalinized, immunologically cross-reactive toxoid (TD) allows us to examine, by default, the role of CT's biochemical properties on the induction of oral tolerance.

MATERIALS AND METHODS

Animals

Adult BALB/c inbred mice between the ages of 6–10 weeks were used. Animals of both sexes were used but within each

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experiment all the mice were of the same gender. The mice were bred and maintained in the Animal Unit, Western General Hospital, Edinburgh.

Antigens

Purified cholera toxin (CT) was obtained from List Biological Laboratories Inc., Campbell, CA. Five times recrystallized ovalbumin (OVA) was obtained from Sigma Chemical Co. Ltd, Poole, Dorset and the immunopurified, formalinized cholera toxoid (TD) was the kind gift of Dr R. O. Thompson of the Wellcome Laboratories, Beckenham, Kent.

Induction, elicitation and measurement of systemic DTH

DTH was induced by intradermally injecting groups of eight mice into the footpad with 50 μl of the appropriate antigen in complete Freund's adjuvant (CFA: Bacto H37 Ra; Difco Ltd, West Moseley, Surrey). After 2 weeks, systemic DTH was assessed by measuring the thickness of the animals' contralateral hind footpad before and 24 hr after the intradermal injection of the challenge antigen. The volume of the challenge inoculate was also 50 μl and the measurement was made using an engineers' caliper (Pocotest-A; Carobronze Ltd, London). Results are expressed as the increment in footpad thickness in mm over this 24-hr period.

Measurement of systemic humoral antitoxin immunity

Mice were bled for antitoxin antibody level estimation 7 and 13 days after immunization and 8 days after footpad challenge for measurement of systemic DTH (Day 22 of the experiment). Serum samples were stored at $-70°$ until assayed. Samples from each experiment were batched and tested at the same time in isotype-specific antitoxin antibody enzyme-linked immunosorbent assays (ELISAs). On each microtitre plate, a negative serum and a positive, hyperimmune serum, both obtained from adult BALB/c mice, were run as calibrators.

Isotype-specific antitoxin antibody ELISAs

EIA microtitration plates (M129A; Dynatech Ltd, Billinghurst, Sussex) were coated at 4° for 16 hr with 5 μg/ml CT in 0·05 M carbonate buffer, pH 9·6. After washing with PBS, pH 7·6, containing 0·05% Tween 20 (BDH Ltd, Poole, Dorset), the plates were incubated with serum samples (1 : 200 for IgG and 1 : 100 for IgM) or dilutions of the control mouse sera in saline with 0·05% Tween 20. All samples and controls were run in duplicate. After 2·5 hr, the plates were washed again and incubated with goat anti-mouse immunoglobulin, isotype-specific, alkaline phosphatase-conjugated antisera (1 : 5000 for anti-IgG (heavy and light chain) and 1 : 2000 for anti-μ chain; Jackson Immunoresearch, Avondale, PA) for a further 3·5 hr, also at room temperature. Finally, *p*-nitrophenylphosphate substrate (Sigma Chemical Co. Ltd) in a 10% diethanolamine buffer, pH 8·6, was added for approximately 30 min at room temperature. In the IgA antitoxin ELISA, the microtitration plates were coated with 5 μg/ml CT in carbonate buffer for 2 hr at 37°. Serum samples were diluted 1 : 20 and incubated on the plate for 16 hr at 4°. Bound antibody was detected by serial incubations with rabbit anti-mouse α chain antibody (1 : 1000; Litton Bionetics Inc, Kensington, MD) followed by alkaline phosphatase-conjugated goat anti-rabbit IgG antiserum (1 : 1000; Northeast Biomedical, Uxbridge, Middlesex). Both these incubations were for 5 hr at room temperature. Plates were washed between each stage and the substrate used was as above.

Assays were run until absorbance readings reached 1·400 and fluctuations between different microtitre plates were minimized by use of the calibrator sera. As data collected in this manner is semi-quantitative, absorbance readings were grouped into various absorbance bands for comparison as shown in Table 1.

Table 1.

Band	Absorbance (405 nm)
0	$<0·038$
1	$<0·075$
2	$<0·150$
3	$<0·300$
4	$<0·600$
5	$<1·200$
6	$\geq 1·200$

Oral immunization

Animals were fed with either alkaline-buffered saline (ABS; PBS pH 7·6 containing 6% w/v $NaHCO_3$), 25 mg OVA, CT or TD, as appropriate, by gavage with a rigid feeding tube with a rounded end. Feeding was performed under light ether anaesthesia, 1 week before the animals were immunized (Day -7 of the experiment). All antigens were dissolved in ABS, and the volume of gavage was 100 μl.

Cyclophosphamide

Animals were pretreated with an intraperitoneal injection of cyclophosphamide (Endoxana; Boehringer Ingleheim, Bracknell, Berkshire), 100 mg/kg, 2 days before they were fed by gavage (Day -9 of the experiment).

Statistics

All experiments were repeated at least three times but only one representative example is shown below.

Means and standard errors of footpad thickness were compared using the student's *t* test and antitoxin antibody levels were compared using the Wilcoxon rank sum test. Statistical significance was reached when $P<0·05$.

RESULTS

Systemic immune response to parenteral cholera toxin

Saline-immunized animals did not produce antitoxin antibodies at any time (Table 2). IgG antitoxin antibodies were detected in CT-primed animals both before and after boosting with TD ($P<0·05$ on Day 13 and $P<0·01$ on Day 22 of the experiment) and in TD-primed animals only after boosting ($P<0·01$). IgA antibodies were only detected in significantly increased amounts on Day 22 of the experiment ($P<0·01$ for CT-primed mice and $P<0·05$ for animals immunized with TD). IgM antitoxin antibody levels were not significantly different from saline-immunized controls in any of the cholera-derived antigen-primed groups at any of the time-points examined.

Table 2. The systemic antitoxin antibody response to parenterally administered cholera-derived antigens

Time (Days)	Imm.*	Footpad challenge	Antitoxin antibodies					
			IgM		IgG		IgA	
			Band†	P‡	Band	P	Band	P
7	SAL	—	3 (2–3)		0 (0–0)		0 (0–0)	
	CT	—	3 (3–4)	NS	0 (0–2)	NS	0 (0–0)	NS
	TD	—	3 (1–3)	NS	0 (0–0)	NS	0 (0–0)	NS
13	SAL	—	2 (1–4)		0 (0–0)		0 (0–0)	
	CT	—	3 (2–4)	NS	2 (2–3)	>0·05	0 (0–0)	NS
	TD	—	3 (2–3)	NS	0 (0–2)	NS	0 (0–0)	NS
22	SAL	TD	3 (1–4)		0 (0–1)		0 (0–0)	
	CT	TD	3 (2–4)	NS	5 (5–5)	<0·01	4 (3–4)	<0·01
	TD	TD	3 (1–4)	NS	3 (2–5)	<0·01	2 (0–3)	<0·05

* Inoculations emulsified in CFA and given on Day 0.

† Represents the median (range) of absorbance reading bands obtained using isotype-specific ELISAs.

‡ Results were compared to those obtained from the ABS-fed animals for each immunization antigen.

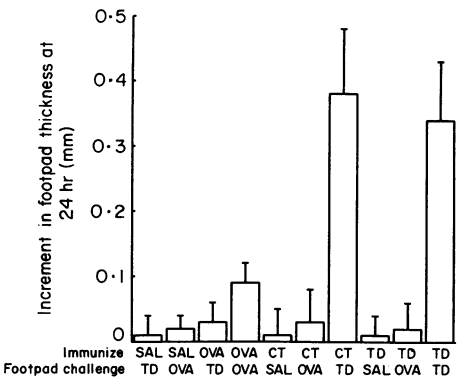

Figure 1. The systemic DTH response to parenterally administered cholera-derived antigens. Animals were immunized with saline or antigens in CFA and footpad challenged 2 weeks later with saline, TD or OVA.

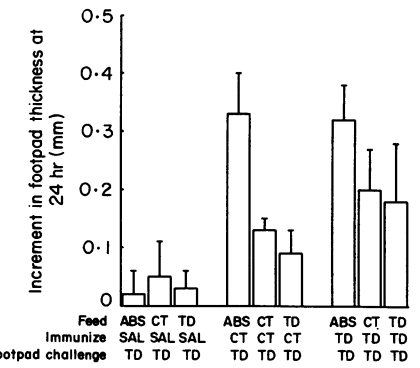

Figure 2. The effect of prefeeding on the induction of systemic immunity. Animals were fed (Day −7) 1 week before immunization (Day 0) and then treated as before. The graphs show the DTH responses obtained on Day 15 of the experiment.

Saline-primed animals did not exhibit any significant footpad swelling 24 hr after challenge with either 100 μg OVA or 5 μg TD (Fig. 1). OVA-primed mice responded with significant DTH only when footpad challenged with 100 μg OVA ($P<0·01$). Both CT- and TD-immunized animals had significant DTH responses when challenged with 5 μg TD ($P<0·001$) but did not significantly differ from saline-primed controls when challenged with 100 μg OVA.

The effect of prefeeding on the induction of systemic immunity

Animals fed either ABS, CT or TD failed to mount significant response when immunized with saline in CFA and challenged with TD. Animals which were sham-fed with only ABS mounted a significant DTH response when immunized and challenged with CT and TD, respectively ($P<0·001$) (Fig. 2). Animals fed either 1 μg CT or 5 μg TD 1 week before immunization still mounted a significant DTH response compared with saline-primed controls ($P<0·01$, in both cases) but had a swelling response which was significantly less than the ABS-fed, CT-immunized and TD-challenged group ($P<0·001$).

Similar results were obtained in TD-immunized mice. ABS-fed controls mounted significant DTH responses ($P<0·001$) which were significantly suppressed in animals fed either CT or TD 1 week before immunization ($P<0·01$, in both cases). Systemic antitoxin antibody responses were similar in sham-fed positive controls and those animals fed either CT or TD 1 week before immunization with CT or TD in CFA (Table 3). All these animals had significantly higher levels of both IgG and IgA antitoxin antibodies when compared to animals primed with

Table 3. The systemic antitoxin antibody responses to orally administered cholera-derived antigens

		Antitoxin antibodies‡			
		IgG		IgA	
Feed*	Immunize†	Band§	*P* value¶	Band	*P* value
ABS	SAL	0 (0–2)		0 (0–0)	
CT	SAL	0 (0–0)	NS	0 (0–0)	NS
TD	SAL	0 (0–0)	NS	0 (0–0)	NS
ABS	CT	5 (5–5)		3 (1–4)	
CT	CT	5 (5–5)	NS	3 (1–4)	NS
TD	CT	5 (5–5)	NS	3 (1–4)	NS
ABS	TD	3 (2–4)		2 (0–3)	
CT	TD	3 (1–5)	NS	2 (0–3)	NS
TD	TD	3 (2–4)	NS	1 (0–3)	NS

* Animals fed 7 days before immunization.

† Inoculations emulsified in CFA and given on Day 0.

‡ Measured using serum obtained on Day 22 of the experiment.

§ Represents the median (range) of absorbance reading bands obtained using isotype-specific ELISAs.

¶ Results were compared to those obtained from the ABS-fed animals for each immunization antigen.

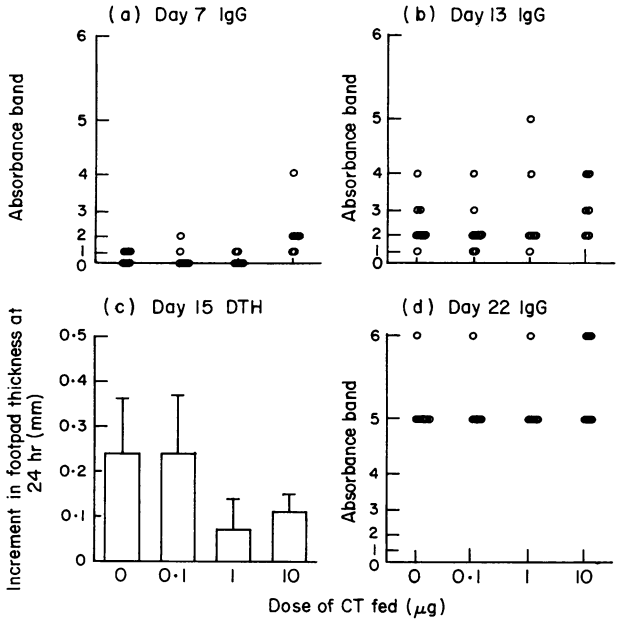

Figure 3. The effect of prefeeding different doses of CT. The above graphs show the IgG antitoxin antibody response on Days 7 (a), 13 (b) and 22 (d) and the DTH response on Day 15 (c) of animals fed as shown and immunised 1 week later with CT (Day 0). All animals were footpad challenged with TD on Day 14 of the experiment.

saline in CFA ($P<0{\cdot}05$ for IgA responses of TD-primed animals, $P<0{\cdot}01$ for the remainder), but as has been noted earlier, both the IgG and IgA antitoxin responses of CT-primed animals were greater than those measured in TD-primed equivalents.

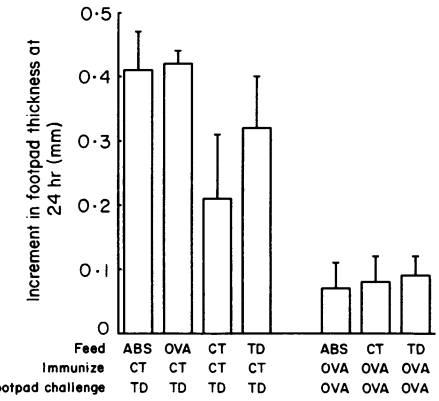

Figure 4. The specificity of oral tolerance to CT and TD. Animals were fed 1 week before immunization and 3 weeks before footpad challenge. The above bars represent the mean increment in footpad thicknesss ±1 SD.

The effect of prefeeding differing doses of cholera-derived antigens

Animals were fed either ABS or 0·1, 1 or 10 μg CT (Day −7) 1 week before immunization with 1 μg CT in CFA (Day 0). Two weeks later (Day 14), they were challenged with 5 μg TD and their DTH responses measured after 24 hr. Animals were bled on Days 7, 13 and 22 of the experiment for assessment of their systemic humoral antitoxin responses.

The systemic immune responses of these animals are shown in Fig. 3. The IgG antitoxin responses of animals fed either 0·1 or 1 μg CT were no different from ABS-fed controls at any time. However, animals fed 10 μg CT had significantly higher IgG antitoxin responses ($P<0{\cdot}02$) on Day 7. After that, they did not significantly differ from sham-fed controls. The IgA responses of CT-fed animals were no different from ABS-fed controls at any time-point examined (data not shown). Neither the IgA or IgG antitoxin responses were significantly altered by feeding TD at any dose at any of the time-points examined (data not shown).

In contrast, animals fed either 1 or 10 μg CT had suppressed DTH responses ($P<0{\cdot}001$ and $P<0{\cdot}01$, respectively) but a dose of 0·1 μg CT did not significantly inhibit the induction of DTH. Similar results were obtained in TD-fed animals. Feeding 5 or 50 μg TD suppressed the induction of DTH but feeding 0·5 μg TD did not.

The specificity of orally induced tolerance

Mice were fed either ABS, 25 mg OVA, CT or TD 1 week before immunization with CT in CFA and challenged 2 weeks after this with TD. ABS- and OVA-fed mice had normal DTH responses but these were significantly suppressed in both CT- and TD-fed animals ($P<0{\cdot}001$ and $P<0{\cdot}05$, respectively) (Fig. 4).

Animals immunized and footpad challenged with 100 μg OVA were fed either ABS, CT or TD 1 week before immunization. Animals fed cholera-derived antigens had DTH responses to OVA which did not significantly differ from sham-fed controls. These experiments demonstrate that oral tolerance is antigen-specific.

The effect of cyclophosphamide-pretreatment on the induction of oral tolerance

In order to explore the possible mechanism by which oral tolerance is induced, animals were pretreated with 100 mg/kg

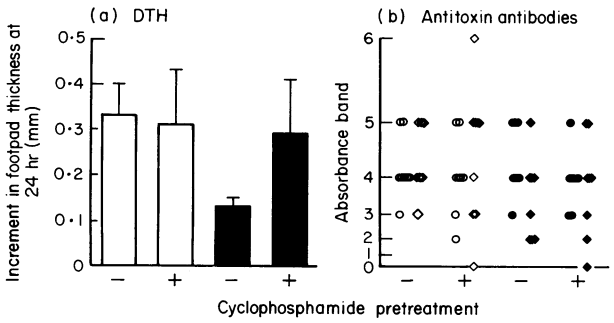

Figure 5. The effect of cyclophosphamide on the induction of oral tolerance to CT. Animals were pretreated with cyclophosphamide (100 mg/kg) or saline 2 days before being fed with ABS (□) or CT (■). Animals were immunized with CT and challenged with TD as before. The above graphs show the (a) DTH responses and the (b) IgG (○) and IgA responses (◇) of these animals.

cyclophosphamide intraperitoneally 2 days before they were fed with either ABS or CT. Animals which were similarly fed were pretreated with a sham injection of saline as a control. Sham-injected animals had normal DTH responses when fed ABS but these responses were significantly suppressed ($P < 0{\cdot}001$) by feeding CT (Fig. 5). Cyclophosphamide pretreatment did not affect the induction of DTH in ABS-fed animals, but abrogated the induction of oral tolerance in CT-fed animals.

Cyclophosphamide did not significantly affect the induction of specific humoral immunity in either ABS- or CT-fed mice.

DISCUSSION

This work has demonstrated that both cholera toxin and a formalinized immunopurified cholera toxoid (which lacks the native toxin's metabolic activities) are capable of inducing simultaneously systemic DTH and humoral immunity when inoculated with adjuvant (CFA). This is the first time both limbs of the systemic effector response to this antigen have been studied together.

We have shown that CT is capable of stimulating higher levels of both IgG and IgA antitoxin antibodies than TD. Unlike other studies which have compared the antigenicity of these two substances, we have used doses of CT and TD with an equivalent cholera toxin B subunit content (Pierce, 1978). This is because the B subunit is considered to be the immunodominant portion of the toxin molecule (Holmgren & Svennerholm, 1979, 1983). Although some enhancement of B-cell priming may accrue from the biochemical properties of CT (Pierce, 1978; Lycke & Holmgren, 1986), there is also evidence to suggest that formalization reduces the antigenicity of B-cell epitopes but leaves T-cell determinants intact (Hua *et al.*, 1985) which would also explain why both cholera-derived antigens are equally capable of boosting a primed response regardless of the agent used for priming (Pierce, 1978; Kay, 1987).

We have also demonstrated that prefeeding animals with either CT or TD inhibits the subsequent induction of specific DTH. Oral tolerance for DTH is found irrespective of which form of cholera-derived antigen is used for feeding or immunization. We have found that neither CT nor TD inhibits the induction of systemic antitoxin antibodies when administered by gavage and the highest dose of CT even enhanced the subsequent specific systemic IgG response. The meager humoral antitoxin response observed after feeding CT reflects that BALB/c mice are of the H-2^d haplotype and as such are poor responders, in terms of antibody, to orally administered CT (Elson & Ealding, 1987). It has been shown previously that systemic cell-mediated and humoral effector mechanisms have differing susceptibilities to being inhibited by orally administered antigens (Mowat *et al.*, 1982) but this is the first time systemic priming for antibody and tolerance for DTH has been reported to occur together after feeding a protein antigen, albeit over a limited dose range. This is further confirmation that there are several mechanisms controlling the mucosal regulation of systemic immunity (Mowat *et al.*, 1982; Mowat, 1987). These results suggest that CT's biochemical actions are not responsible for the induction of oral tolerance but that they may be important for the induction of systemic humoral immunity.

When CT is fed, it induces both local and systemic antibody, antitoxin-containing cells and isotype-specific helper T cells (Pierce, 1978; Elson & Ealding, 1984b; Lange *et al.*, 1984). It has also been demonstrated that feeding CT abrogates the induction of oral tolerance for antibody not only to cholera-related antigens but also other unrelated antigens when they are fed with it (Elson & Ealding, 1984a). Furthermore, nanogram quantities of CT can be used to boost the intestinal humoral immune responses both to related and unrelated antigens without inducing a response to itself (Lycke & Holmgren, 1986). CT's lectin-like qualities may also be contributory in the induction of systemic antibodies, as covalently binding horseradish peroxidase (HRP) to cholera toxin B subunit leads to the induction of anti-HRP antibodies after feeding whereas none are detected before the two substances are bound together (McKenzie & Halsey, 1984). It is therefore possible that the lack of oral tolerance observed after feeding TD is a reflection of the inherent antigenic structure of this protein but that the induction of a systemic immune response to CT is a result of its additional 'toxic' activities.

The induction of oral tolerance for DTH to CT, on the other hand, may reflect a common response controlling the induction of CMI to all soluble protein antigens. In experiments which have examined systemic and local CMI after feeding, the induction of oral tolerance for DTH and intestinal CMI have always been found to be mutually exclusive events (Mowat *et al.*, 1982; Strobel & Ferguson, 1984) and procedures which abrogate the induction of oral tolerance also induce intestinal CMI reactions (Mowat *et al.*, 1982; Mowat & Parrott, 1983; Strobel, Mowat & Ferguson, 1985; Mowat, 1986). Furthermore, when the effect of feeding OVA was studied in a range of genetically different mice, the induction of oral tolerance for DTH was a consistent occurrence (Lamont *et al.*, 1988). While protective local humoral immunity may occur without detriment to the host, the same is not true for CMI which causes both anatomical and physiological changes within the intestinal mucosa with potentially fatal consequences (Ferguson, 1987). Clearly, the down-regulation of DTH confers an evolutionary advantage to the host and the above result demonstrates that this immune response probably occurs to a variety of soluble protein antigens as well as against a variety of genetic backgrounds. We therefore agree with other workers that the prevention of intestinal, antigen-specific DTH reactions represents a major homeostatic role for oral tolerance (Mowat, 1987; Lamont *et al.*, 1988).

It has also been found that the induction of oral tolerance is

cyclophosphamide-sensitive (Mowat *et al.*, 1982; Strobel *et al.*, 1983). T suppressor and B cells are more sensitive to the action of cyclophosphamide than are cytotoxic or T-helper cells (Kaufmann, Hahn & Diamantstein, 1980; Shand & Liew, 1980) and when used in a dose range of 20–200 mg/kg has been shown to inhibit short-lived suppressor cells affecting DTH responses (Attallah, Ahmed & Sell, 1979; Diamantstein *et al.*, 1981). Cyclophosphamide pretreatment has been shown to abrogate the induction of oral tolerance to OVA without damaging the intestinal mucosa (Mowat *et al.*, 1982; Strobel *et al.*, 1983) and we have shown that cyclophosphamide pretreatment of CT-fed animals abrogated the induction of oral tolerance to DTH but left humoral immunity unchanged. Experiments using the drug, deoxyguanosine, and cell-transfer protocols have suggested that suppressor T cells control the induction of DTH (Mowat, 1986; Miller & Hanson, 1979) and our results are consistent with this. Therefore, it may be that our results reflect not only a common response to oral administered soluble protein antigens but also a common regulatory mechanism controlling systemic DTH.

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