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Depletion of suppressor T cells by 2′-deoxyguanosine abrogates tolerance in mice fed ovalbumin and permits the induction of intestinal delayed-type hypersensitivity

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SUMMARY

We have re-examined the role of suppressor T cells (T_s) in regulating immune responses to fed proteins by investigating the effect of 2′-deoxyguanosine (dGuo) on systemic and intestinal immunity in mice fed ovalbumin (OVA). Administration of dGuo for 10 days abrogated the suppression of systemic delayed-type hypersensitivity (DTH) and antibody responses normally found after feeding OVA, and also prevented the generation of OVA-specific T_s. In parallel, mice given dGuo and fed OVA developed sensitization to OVA in the gut-associated lymphoid tissues (GALT) after oral challenge with OVA and had increased intraepithelial lymphocyte (IEL) counts and crypt cell production rates (CCPR) in the jejunal mucosa, indicating the presence of a local DTH response. These findings confirm the importance of T_s in preventing hypersensitivity to dietary protein antigens and suggest that enteropathies associated with food hypersensitivity are due to a defect in T_s activity.

INTRODUCTION

Individuals with small intestinal damage and malabsorption associated with food hypersensitivity exhibit systemic and local immunity to dietary antigens, but the pathogenesis of these disorders is not yet understood (Walker-Smith, 1982; Mowat, 1984). The usual outcome of feeding proteins to naive animals is a state of systemic tolerance, but if this oral tolerance can be abrogated, active local delayed-type hypersensitivity can be induced in the small intestine on refeeding the antigen (Mowat & Ferguson, 1981; Mowat & Parrott, 1983). As the mucosal changes associated with the local DTH response, including increases in intraepithelial lymphocyte count and crypt cell production rate, are qualitatively similar to those found in food-sensitive enteropathy (FSE), it has been proposed that FSE reflects a breakdown in oral tolerance to dietary protein antigens (Mowat & Ferguson, 1981). Therefore, investigation of the mechanisms underlying oral tolerance in experimental animals should help clarify the pathogenesis of FSE in man.

The tolerance of cell-mediated immunity in mice fed protein antigens is normally ascribed to the action of suppressor T cells (Richman *et al.*, 1978; Miller & Hanson, 1979; Challacombe & Tomasi, 1980; Mowat *et al.*, 1982; Mowat, 1985), and these T_s are sensitive to the action of cyclophosphamide (CY) (Mowat *et al.*, 1982; Hanson & Miller, 1982). In previous work, we have shown that elimination of T_s by CY not only abrogates oral tolerance to ovalbumin in mice, but also allows the induction of mucosal DTH and have suggested that FSE is due to a lack of T_s in genetically susceptible individuals (Mowat & Ferguson, 1981; Mowat *et al.*, 1982). Nevertheless, the use of CY as a model for T_s deficiency *in vivo* has the disadvantage that CY is a potent pharmacological agent with a wide range of actions, including a potential for damaging the intestine (Sobhon, Wanichanon & Sretarugsa, 1977). Therefore, it would be important to develop an animal model of FSE using agents with a more physiological action, but that are selective for T_s. 2′-Deoxyguanosine is a product of normal purine metabolism, the accumulation of which in patients with purine nucleoside phosphorylase (PNP) deficiency is probably responsible for the immune disorder observed in this condition (Ammann, 1978). Recently, it has been shown that administration of exogenous dGuo selectively prevents the generation of T_s *in vivo* (Dosch *et al.*, 1980; Varey *et al.*, 1983; Bril *et al.*, 1984, 1985), whereas the effector cells of DTH (T_{DTH}) are resistant to dGuo (Bril *et al.*, 1984, 1985). As intestinal epithelial cells also do not contain the kinase enzymes that are required to produce toxic nuclotides from dGuo (Carson, Keeg & Seegmiller, 1977), dGuo offers a potential means of selectively depleting orally induced T_s without affecting handling of antigen in the gut. Therefore, in this study, we have examined whether dGuo interferes with the induction of tolerance to fed OVA by depleting T_s. In addition, we have used dGuo to confirm our hypothesis that elimination of T_s allows potentially damaging DTH to develop in the intestine of protein-fed animals.

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MATERIALS AND METHODS

Animals

Female BALB/c mice were first used at 8–10 weeks of age.

2'-Deoxyguanosine

2'-Deoxyguanosine (Sigma, Poole, Dorset) was dissolved in distilled water at 5 mg/ml, and mice were injected with 1 mg daily by the intraperitoneal route.

Ovalbumin

Ovalbumin (Sigma Fraction V) was dissolved in distilled water for use. Heat-aggregated OVA was prepared by the method of Titus & Chiller (1981) as described previously (Mowat & Parrott, 1983).

Induction of oral tolerance

Mice were fed 25 mg ovalbumin or distilled water by gavage and immunized 10 days later with 100 μg OVA in complete Freund's adjuvant (CFA) (Bacto H37Ra, Difco, West Molesey, Surrey) injected into one footpad. Twenty-one days after immunization, mice were assessed for systemic DTH and antibody responses. Mice treated with dGuo were injected daily from the time of feeding until immunization, while control groups were fed water and injected daily with dGuo or received daily injections of water after being fed water.

Systemic delayed-type hypersensitivity

Immunized mice were challenged with an intradermal injection of 100 μg heat-aggregated OVA into the opposite footpad, and the DTH response assessed as the increment in footpad thickness 24 hr later. OVA-specific DTH was then calculated by subtracting the response to OVA in unimmunized mice.

Systemic IgG antibody responses

Mice were bled from the retro-orbital plexus under light ether anaesthesia, and the serum assayed for antibodies to OVA by an ELISA. Flat-bottomed microtitre plates (Titertek, Flow Labs, Irvine, Ayrshire) were coated with 1 mg/ml OVA overnight at 4° and then washed three times in PBS containing 0·1% NaN_3 and 0·05% Tween-20 (Sigma). After incubation with serum samples diluted 1/1000 for 3 hr at 37°, plates were washed three times and then incubated with 1/1000 sheep anti-mouse IgG (Serotec, Bicester, Oxon) overnight at 4°. The plates were washed and 1/1000 rabbit anti-goat IgG conjugated to alkaline phosphatase (Miles-Yeda Ltd, Slough, Berks) added for 3 hr at 37°. One mg/ml phosphatase substrate (Sigma 104) was added for 30 min at room temperature, and the reaction stopped with 3 M sodium hydroxide. The absorbance at 405 nm (OD_{405}) was read using a Titertek Uniskan Elisa Reader (Flow Labs) and corrected for non-specific binding by subtracting the OD_{405} obtained in the absence of specific anti-OVA antibody. Assays were performed in triplicate, and in each assay dilutions of a standard mouse anti-OVA serum were included for reference.

Induction of suppressor cells by feeding OVA

T_s were generated by feeding OVA as described previously (Mowat, 1985). Mice were fed 25 mg OVA and their spleens removed 7 days later to obtain suppressor cells. Viable cells (10^8) were then transferred i.v. into normal BALB/c recipients, which were immunized immediately with 100 μg OVA/CFA and assessed for systemic immunity 21 days later. In this experiment, one group of mice received spleen cells from donors that had received 1 mg/day dGuo during the period between feeding OVA and harvesting spleen suppressor cells, while control spleen cells were obtained from normal BALB/c donors.

Induction of DTH in the intestinal mucosa

Mice were fed 2 mg OVA and were then challenged 28 days later with 0·1 mg OVA/day incorporated in their drinking water. One group of mice was given 1 mg OVA/day incorporated in their drinking water. One group of mice was given 1 mg dGuo/day i.p. for 12 days after the first feed of OVA, while control mice received dGuo only. All mice were challenged orally with OVA for 10 days and mucosal DTH was then assessed as described previously (Mowat & Ferguson, 1981; Mowat & Parrott, 1983). Briefly, mice were killed at intervals of 20–100 min after the injection of 7·5 mg/kg colchicine (Sigma) i.p. and pieces of jejunum taken 10 cm from the pylorus. After fixation in 75% ethanol/25% acetic acid, these were stained in bulk with Schiff reagent (Difco), dissected under microscopy, and villus and crypt lengths measured by micrometery. Crypt cell production rates (CCPR) were calculated by linear regression analysis of the number of accumulated metaphases per crypt at each time after colchicine. Ten villi and crypts were counted on each specimen. Intraepithelial lymphocytes were counted on haematoxylin and eosin stained sections by the method of Ferguson & Murray (1971) and are expressed as IEL/100 epithelial cells.

Sensitization to OVA in mesenteric lymph nodes

The development of sensitization in the MLN was examined by assessing the migration inhibition of MLN lymphocytes in the presence of OVA, as described previously (Mowat & Ferguson 1982a, b). MLN were pooled from three to four mice per group on completion of secondary oral challenge with OVA and 10^8 cells/ml pelletted into 10 μl capillary tubes (Drummond Microcaps). The tubes were placed in the wells of leucocyte migration inhibition plates (Sterilin Ltd, Teddington, Middlesex) and incubated in 0·45 ml RPMI-1640 (Gibco, Paisley, Renfrewshire) with or without 0·1 mg/ml OVA for 21 hr at 37° in 5% CO_2. The areas of migration were then drawn using an inverted microscope and measured by planimetry. The migration index (MI) was calculated as follows:

$$MI = \frac{\text{area of migration in presence of OVA}}{\text{area of migration in medium only}}.$$

Statistics

Results expressed as means $\pm$ one standard deviation were compared by Student's t-test, while linear regression data were compared by covariance analysis. Values shown as percentage suppression were obtained as follows:

$$\% \text{ suppression} = \frac{(\text{response in controls} - \text{response in tolerant mice})}{\text{control response}} \times 100.$$

RESULTS

Effect of 2'-deoxyguanosine on oral tolerance to OVA

As anticipated, mice fed 25 mg OVA had significant suppression of both systemic DTH (Fig. 1) and serum IgG antibody responses (Fig. 2) compared with immunized control mice (89% suppression $P < 0·025$ and 35% suppression $P < 0·02$, respectively). In contrast, mice receiving dGuo after feeding OVA did not have significant tolerance of DTH or antibody production, and these responses were significantly greater than those of mice only fed OVA ($P < 0·05$ and $P < 0·02$, respectively). dGuo on its

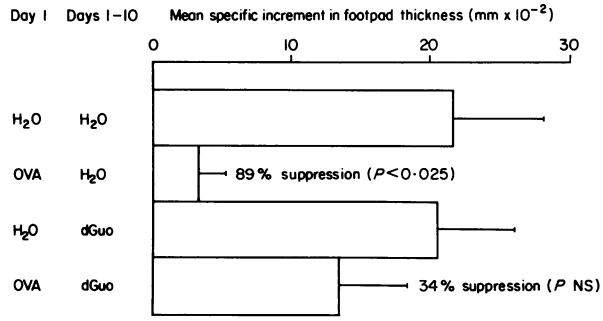

Figure 1. Effect of dGuo on oral tolerance to ovalbumin. Systemic DTH responses 3 weeks after immunization with OVA/CFA in mice fed 25 mg OVA and given 10 daily injections of dGuo i.p. Results shown are mean specific increments in footpad thickness + 1 SD for groups of six to eight mice.

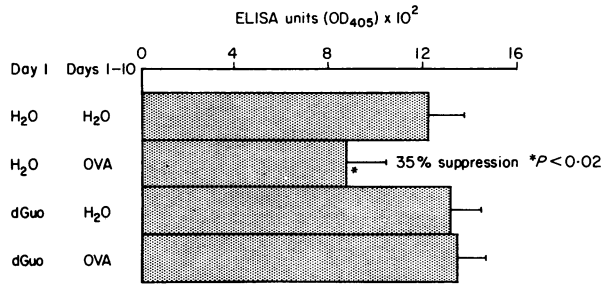

Figure 2. Effect of dGuo on oral tolerance to ovalbumin. Serum IgG antibody responses measured by ELISA 3 weeks after immunization with OVA/CFA in mice fed 25 mg OVA and given 10 daily injections of dGuo i.p. Results shown are mean specific OD_{405} + 1 SD for groups of six to eight mice.

own had no effect on the immune responses of mice compared with controls.

Depletion of suppressor cells by dGuo

As dGuo abrogated oral tolerance in mice fed OVA and has been shown to eliminate T_s in other systems (Dosch *et al.*, 1980; Varey *et al.*, 1983; Bril *et al.*, 1984, 1985), we examined the induction of T_s in dGuo-treated mice after feeding OVA. Spleen cells were obtained from mice fed OVA 7 days earlier, and immunized recipients were examined for systemic immunity 3 weeks later. Recipients of cells from OVA-fed donors had significant suppression of systemic DTH compared with recipients of normal spleen cells (Fig. 3; 57% suppression $P<0.005$), while spleen cells from mice given dGuo after feeding OVA did not suppress DTH reactivity in recipients. [The suppressor cells were eliminated by treatment with anti-Thy 1.2 + complement (data not shown).] The serum antibody responses in recipients of OVA-fed spleen cells showed no suppression (data not shown).

Thus, the ability of dGuo to prevent the induction of oral tolerance of DTH after feeding OVA was associated with defective induction of OVA-specific suppressor cells.

Ability of dGuo to allow priming of gut-associated lymphoid tissues after feeding OVA

In mice treated with CY or oestrogen, abrogation of oral tolerance is associated with the induction of active immunity in

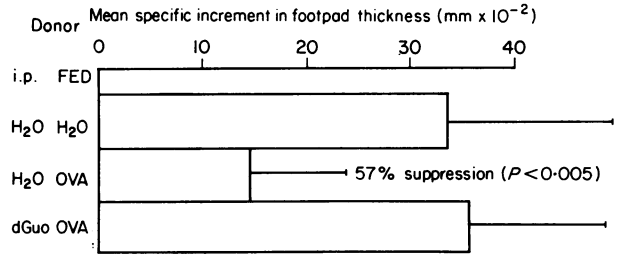

Figure 3. Effect of dGuo on the generation of suppressor cells after feeding ovalbumin. Systemic DTH responses in recipients of OVA-fed spleen cells 3 weeks after immunization with OVA/CFA. Results shown are mean specific increments in footpad thickness + 1 SD for groups of five to six mice.

the intestine and GALT (Mowat & Ferguson, 1981; Mowat & Parrott, 1983). Therefore, we sought evidence for local immunity in the GALT of mice fed and challenged with OVA after treament with dGuo by assessing the inhibition of migration of MLN lymphocytes in the presence of OVA. This assay has been shown to correlate with regional DTH in the GALT of OVA-fed mice (Mowat & Ferguson, 1982a, b).

MLN lymphocytes from mice fed OVA and treated with dGuo showed significant inhibition of migration in the presence of OVA when tested after 10 days of secondary oral challenge with OVA (Fig. 4; MI = 0·59 ± 0·12, $P<0.02$). In contrast, MLN lymphocytes from mice fed and challenged with OVA alone or given dGuo alone showed no inhibition of migration in the presence of OVA (MI = 1·16 ± 0·26 and 1·03 ± 0·17, respectively). Thus, dGuo has allowed the induction of local immunity in the GALT of mice fed and challenged orally with OVA.

Induction of mucosal delayed-type hypersensitivity to OVA after treatment with dGuo

As dGuo-treated OVA-fed mice had evidence of immunological priming in the GALT, we investigated whether a DTH response

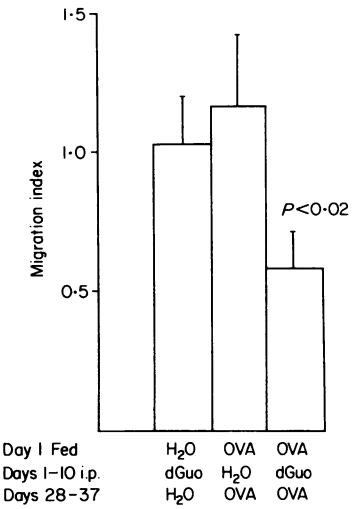

Figure 4. Induction of regional immunity to fed ovalbumin by treatment with dGuo. MLN cells were pooled from groups of three to four mice after 10 days oral challenge with OVA and migrated in the presence of 0·1 mg/ml OVA. Results shown are combined from two experiments and are the mean migration index + 1 SD for eight wells per group in each experiment.

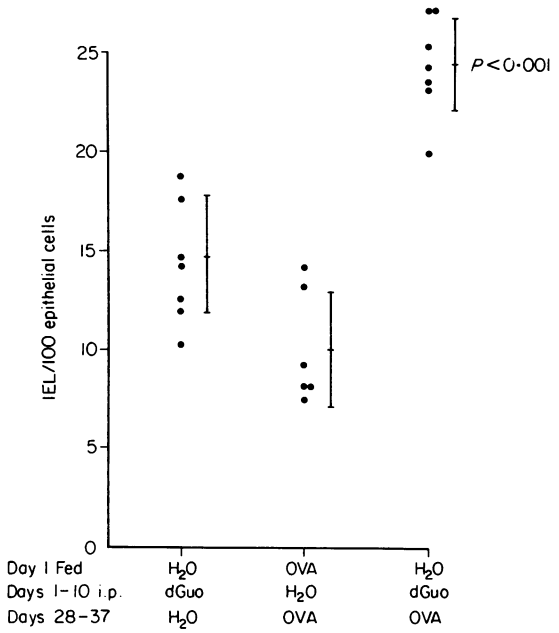

Figure 5. Induction of mucosal DTH to fed ovalbumin by treatment with dGuo. Intraepithelial lymphocyte counts in the jejunum of mice fed OVA and given dGuo, on completion of 10 days oral challenge with OVA. Results shown are mean IEL/100 epithelial cells ± 1 SD.

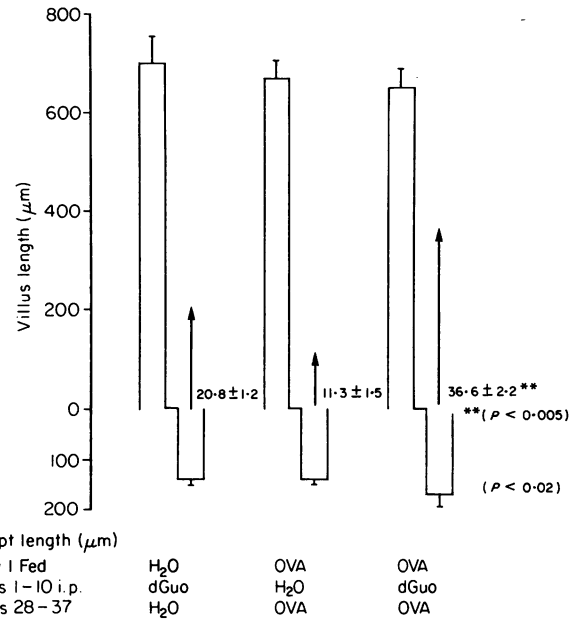

Figure 6. Induction of mucosal DTH to fed ovalbumin by treatment with dGuo. Mucosal architecture in the jejunum of mice fed OVA and given dGuo, on completion of 10 days oral challenge with OVA. Bars represent mean villus and crypt lengths, and arrows represent CCPR. Results shown are means ± 1 SD for groups of six to seven mice.

could also be induced in the mucosa of mice fed OVA and treated with dGuo.

In this experiment, mice were again challenged for 10 days with OVA in the drinking water 28 days after being fed OVA, and mucosal DTH was assessed by examining for increases in IEL count, crypt length and CCPR in the jejunum. As shown in Fig. 5, after oral challenge with OVA, mice fed OVA and treated with dGuo had a significant increase in IEL count compared with mice fed and challenged with OVA alone or receiving dGuo alone (24·4 ± 2·5 *vs* 10·1 ± 2·9 and 14·8 ± 3·0 IEL/100 epithelial cells, respectively, $P < 0{\cdot}001$). In addition, mice fed OVA and given dGuo had crypt lengths (171·4 ± 23·8 μm, $P < 0{\cdot}02$) and CCPR that were greater than those of mice fed OVA alone (139·3 ± 7·9 μm) or given dGuo alone (144·5 ± 9·6 μm) when challenged orally with OVA (Fig. 6). Villus atrophy was not observed in any group.

Thus, dGuo-treatment of mice fed OVA allows the induction of active DTH in the intestinal mucosa after a secondary oral challenge with antigen.

DISCUSSION

These results show that administration of dGuo prevents the generation of T_s and abrogates the induction of systemic tolerance normally found in mice fed OVA. Furthermore, when dGuo-treated mice are fed and challenged orally with OVA, an active DTH response develops in the small intestine and its lymphoid tissues. As the accompanying mucosal alterations are qualitatively similar to those found in humans with food-sensitive enteropathy, our study confirms the importance of T_s in regulating immune responses to dietary protein antigens.

There are no previous reports of the effect of dGuo on immunity to orally administered antigens, but studies of dGuo and systemic immunity support our finding that dGuo enhances active immunity by eliminating T_s. dGuo is thought to be the toxic metabolite responsible for the depletion of T lymphocytes associated with PNP deficiency (Ammann, 1978) and, more recently, several workers have shown that exogenous dGuo selectively prevents the generation of T_s *in vivo* and *in vitro* (Gelfand, Lee & Dosch, 1979; Dosch *et al.*, 1980; Varey *et al.*, 1983; Bril *et al.*, 1984, 1985). Furthermore, as we have shown here, dGuo can enhance active immune responses after immunization, because it is not toxic to the effector T cells of cytotoxicity or DTH (Dosch *et al.*, 1980; Varey *et al.*, 1983; Bril *et al.*, 1984, 1985) and does not interfere with antibody synthesis (Dosch *et al.*, 1980; Hanglow & Lydyard, 1985). The mechanism of toxicity of dGuo on T_s is controversial. It has been proposed that all dividing T lymphocytes are susceptible to dGuo because cellular dGuo kinase converts dGuo to dGTP with resulting inhibition of the enzymes required for DNA synthesis (Carson *et al.*, 1977; Dosch *et al.*, 1980; Cohen *et al.*, 1980; Spaapen *et al.*, 1984). Therefore, as dGuo only inhibits the generation of T_s and does not prevent the expression of mature effector T_s (Lelchuk, Cooke & Playfair, 1982; Bril *et al.*, 1985), it has been assumed that dGuo eliminates T_s merely because of the known requirement for cell division by T_s precursors (Rollinghoff *et al.*, 1977). However, recently it has been shown that the precursors of effector T cells also need to divide but are not sensitive to dGuo (Bril *et al.*, 1985). Toxicity of dGuo is critically dependent on the ability of many different purine degradation enzymes to produce toxic metabolites (Carson *et al.*, 1977; Cohen *et al.*, 1980; Spaapen *et al.*, 1984), and thus it is possible that dividing T_s precursors are particularly sensitive to dGuo because of a unique complement of purine salvage enzymes. It would be of interest to analyse these enzymes in cloned populations of T_s in order to resolve this issue.

In addition to its selective effect on T_s, dGuo has one theoretical advantage for studies of immunity to dietary antigens: that it should not damage the intestinal mucosa. In our previous studies, the modulation of immunity to fed OVA by administration of CY was complicated by its potential for damaging the intestinal epithelium (Sobhon *et al.*, 1977). Although studies of intestinal uptake of OVA indicated that CY was not acting in this manner (Strobel *et al.*, 1983), dGuo offers a more selective means of modifying only the immune system in protein-fed animals. As intestinal epithelial cells have little dGuo kinase and are rich in 5'nucleotidase (Carson *et al.*, 1977), dGuo will not be converted to toxic dGTP and the mucosa should be resistant to dGuo toxicity. We are currently examining the effects of dGuo on intestinal integrity and function with a view to confirming this directly.

There are two important practical implications of our study. Firstly, patients with PNP deficiency occasionally survive into adulthood and have been shown to develop autoimmune diseases (Ammann, 1978). This observation is initially surprising in view of the immunodeficiency normally associated with PNP deficiency, but the results presented here would indicate that a selective deficiency of T_s is the mechanism responsible for autoimmunity in these patients. It would be of interest to examine directly for T_s activity in patients with PNP deficiency. The most significant aspect of this study is that it confirms that T_s are critically important for determining the immunological consequences of feeding proteins. As we have shown here, T_s are normally present in mice fed OVA (Richman *et al.*, 1978; Miller & Hanson, 1979; Challacombe & Tomasi, 1980; Hanson & Miller, 1982), but when these T_s were eliminated, the systemic tolerance that usually occurs was abrogated. In addition, the elimination of T_s and abrogation of oral tolerance by dGuo was accompanined by the induction of an active DTH response in the intestine and its lymphoid tissues. This extends our previous reports, which showed that intestinal DTH can be induced in mice fed OVA after tolerance has been abrogated with CY or oestrogen (Mowat & Ferguson, 1981; Mowat & Parrot, 1983) or when tolerance is defective in immature mice (Strobel & Ferguson, 1982, 1984). However, we did not examine directly for T_s activity in these studies and our present findings confirm that elimination of T_s is one mechanism that allows the development of intestinal immunity to dietary proteins. Under these circumstances, the mucosal alterations associated with the local DTH response are qualitatively similar to those found in infants with enteropathy due to food hypersensitivity. In addition, experimental induction of systemic tolerance of food proteins prevents the development of enteropathy and malabsorption caused by food hypersensitivity in neonatal piglets (Miller *et al.*, 1984). Taken together, these findings suggest that FSE reflects a defect in a potent homeostatic mechanism, which normally prevents intestinal DTH to dietary proteins, and supports our hypothesis that a deficiency of T_s is responsible for the induction of active immunity to food proteins in FSE (Mowat & Ferguson, 1981). Furthermore, although food hypersensitivity has not been studied in PNP deficiency, and there is no evidence to suggest that patients with FSE have major abnormalities in purine metabolism, our study raises the intriguing possibility that genetic or maturational defects in T_s enzymes could be one of the factors underlying the susceptibility to FSE in infants.

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