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# Hypersensitivity in the small intestinal mucosa

## V. INDUCTION OF CELL-MEDIATED IMMUNITY TO A DIETARY ANTIGEN

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## SUMMARY

Feeding of a protein antigen to adult mice results in reduced humoral and cell-mediated immune (CMI) responses when that antigen is subsequently presented, and also causes activation of suppressor cells in the gut-associated lymphoid tissues (GALT). We have attempted to abrogate this tolerance to fed antigen by pretreating mice with 100 mg/kg cyclophosphamide before oral immunization and challenge with ovalbumin. Cyclophosphamide-pretreated mice did not develop serum haemagglutinating antibodies, nor systemic CMI (as assessed by skin testing) after ovalbumin feeding. However, evidence that CMI had been induced in the GALT was provided by the significant inhibition of migration of mesenteric lymph node cells from cyclophosphamide-pretreated animals, but not from other control groups, in the presence of ovalbumin. Our previous work on CMI reactions in the small intestine has shown that the cell production rate in the crypts of Lieberkuhn and the intraepithelial lymphocyte count are reliable although indirect measures of mucosal CMI. Cyclophosphamide-pretreated, ovalbumin-immunized animals, which had been fed 0·1 mg ovalbumin daily for 10 days before killing, had increased crypt cell mitoses, and increased intraepithelial lymphocyte counts, indicating the presence of mucosal CMI response to ovalbumin. Mechanisms whereby cyclophosphamide pretreatment leads to abrogation of tolerance and induction of mucosal CMI are discussed.

## INTRODUCTION

Intestinal mucosal immune responses to food antigens have been implicated in the small intestinal injury of coeliac disease and other malabsorption syndromes with food protein intolerance (Ferguson & Mowat, 1980). Allergy (hypersensitivity) to food antigens may also cause atopic disease in infants (Soothill, 1977). Thus, since the primary pathology in these and similar diseases may be abrogation of a normal, harmless immune response to dietary antigen, there is a need to study mechanisms underlying the induction of immunity to fed antigens.

Feeding of antigen induces a secretory antibody response (reviewed in Heremans, 1974), but little is known of the factors which will induce cell-mediated immunity to fed antigens. Induction of systemic tolerance to a fed antigen has been well documented (Chase, 1946; Thomas & Parrott, 1974; Hanson *et al.*, 1977; Richman *et al.*, 1978) and this may be associated with the ability of the gut-associated lymphoid tissues (GALT) to generate suppressor T cells in response to orally administered antigen (Ngan & Kind, 1978; Mattingly & Waksman, 1978). We have investigated the hypothesis that induction of mucosal hypersensitivity may be the result of deficient GALT suppressor mechanisms by orally immunizing animals after pretreatment with cyclophosphamide. Administration of cyclophosphamide to mice in the dose 100 mg/kg enhances cell-mediated

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immune reactions without an appreciable effect on antibody synthesis, via suppressor cell inhibition (Askenase, Hayden & Gershon, 1975).

The small intestine and its lymphoid elements do not lend themselves to the direct identification and measurement of delayed hypersensitivity responses occurring locally in the gut wall, and indirect measures of such reactions must be employed.

Our previous studies on two models of local cell-mediated immunity (CMI), allograft rejection and graft-versus-host reaction (GvHR), have shown that local CMI reactions alter the cell proliferation kinetics of the intestinal epithelium, and also increase the numbers of lymphocytes within the intestinal epithelium. We found that an increase in the mitotic activity of crypt cells, the crypt cell production rate (CCPR), was the earliest and most sensitive change (MacDonald & Ferguson, 1976, 1977; Mowat & Ferguson, 1981). Thus the parameters used in this study to monitor the immune reaction to orally fed ovalbumin (OVA) were serum haemagglutinating antibody levels, direct migration inhibition of lymph node cells in the presence of antigen and, in the mucosa itself, the CCPR and the intraepithelial lymphocyte (IEL) count, all of these after the animals had been challenged by feeding of ovalbumin for 10 days.

## MATERIALS AND METHODS

*Animals.* Female BALB/c mice aged 8–10 weeks were used throughout, and these were maintained on an egg protein-free diet from birth under conventional conditions.

*Cyclophosphamide treatment.* Cyclophosphamide (Endoxana, WB Pharmaceuticals) was dissolved in saline and mice were given 100 mg/kg intraperitoneally 2 days before primary oral immunization.

*Oral immunization.* For primary immunization, mice were fed a single dose of 2 mg ovalbumin (Sigma fraction V) in saline by intragastric tube. Twenty-eight days later, OVA (2 mg/100 ml) was added to drinking water, and since mice drink approximately 5 ml per day, each animal would have received an estimated 0·1 mg OVA daily. All animals were killed after 10 days of ovalbumin feeding.

*Parenteral immunization.* As positive controls for the haemagglutination assays, mice were immunized with 2 mg OVA in 0·2 ml Freund's complete adjuvant (FCA; DIFCO), which contained 1 mg dried Mycobacteria/ml. Ovalbumin (0·5 mg) was injected into each of the four footpads and animals were given a second OVA dose 30 days later, 1 mg OVA in saline intraperitoneally. Sera for antibody assays were obtained at the time of killing, 1 week after secondary immunization, by exsanguination from the axillary vessels. As positive controls for tests of delayed hypersensitivity, further animals were immunized with 0·1 mg OVA in 0·2 ml FCA, distributed between all four footpads.

*Haemagglutination assay.* Sera were tested for the presence of anti-OVA antibodies by haemagglutination of OVA-coated sheep red blood cells (SRBC). Fresh heparinized SRBC were washed three times in saline at room temperature and 100 μl of washed, packed cells mixed with 0·7 ml saline and 100μl of OVA solution (15 mg/ml). One millilitre of 0·01% chromic chloride (Analar; BDH Ltd) at pH 5 was then added dropwise with continuous agitation and the mixture allowed to stand at room temperature for 10 min before the reaction was stopped with phosphate-buffered saline (PBS) at pH 7·2. The coated cells were then washed twice in PBS and resuspended at 1%. All sera were inactivated at 56°C for 30 min and absorbed with 10% uncoated SRBC for 1 hr before use. Twenty-five microlitres of serum were doubly diluted in round-bottomed microtitre plates (Titretek), 25 μl coated SRBC added to each well and the plates incubated for 90 min at room temperature. The titre was taken as the last well to show complete agglutination.

*Skin testing for delayed hypersensitivity.* The presence of systemic DTH was assessed in the various groups of immunized mice by a conventional intradermal skin test. Mice were injected with 100 μg OVA in 0·05 ml saline intradermally on both shaven flanks, and double skinfold thickness measured with calipers immediately before and 24 hr after injection. The results were expressed as mean increase in skin thickness in mm at 24 hr. Orally immunized mice were tested in this way both 21 days after primary oral immunization and at the end of the 10 days of feeding, while parenterally immunized mice were tested 21 days after sensitization.

*Cell migration inhibition.* Draining lymph nodes from immunized mice (mesenteric from orally immunized mice or axillary and popliteal from parenterally immunized mice) were removed, washed in RPMI 1640 medium (Flow Laboratories) and trimmed of surrounding material, before being gently cut up and passed through a fine wire-mesh filter. After one passage through a 25-g needle, the cells were washed three times in RPMI 1640 and finally adjusted to approximately $100 \times 10^6$ cells/ml in RPMI 1640 supplemented with 2 mmol/l glutamine (Flow Laboratories), 100 u/ml penicillin G and 100 $\mu$g/ml streptomycin (Flow Laboratories) and buffered with 10 mmol/l HEPES buffer (Flow Laboratories). Glass capillary tubes, 10 $\mu$l capacity (Drummond Microcaps), were filled with the cell suspension, sealed at one end with Cristaseal Wax (Hawksley & Sons Ltd) and spun at 400 *g* to form a cell pellet. The capillaries were then cut just above the cell interface and embedded in silicon grease (Edwards Ltd) in the wells of macrophage inhibition test plates (Sterilin Ltd) and the wells filled with either 0·45 ml supplemented RPMI 1640 medium alone, or medium containing 0·1 mg/ml OVA. After sealing the wells with round glass coverslips, the plates were incubated at 37°C for 20 hr and the areas of migration drawn using a drawing tube attached to a dissecting microscope. Areas were measured by a planimeter (Allbrit) and expressed in $cm^2$. For each test, the migration index (MI) of the cells incubated with OVA was given as area of migration with antigen/area of migration in control wells.

*Small intestinal epithelial cell kinetics.* Villus heights, crypt depths and crypt cell production rates were measured for groups of animals by the technique first described by Clarke (1970) and previously used by us for experiments on allograft rejection of gut (MacDonald & Ferguson, 1977). Briefly, on the day of killing, all groups of animals were given 7·5 mg/kg colchicine (BDH Ltd) to arrest metaphase production, and individual animals were killed at various intervals (from 20–100 min) afterwards. Pieces of jejunum, 10 cm from the pylorus, were removed and fixed in 75% ethanol/25% acetic acid for 6 hr, and stained with modified Feulgen stain (Schiff reagent; DIFCO). The villus height and crypt depth were expressed in microns and the crypt cell production rate (CCPR) is given as the net accumulation of metaphases per crypt per hour.

*Intraepithelial lymphocyte counts.* Pieces of jejunum were fixed in 10% buffered formol saline, paraffin embedded, 5-$\mu$m sections cut and stained with haematoxylin and eosin. A differential count was made of cell types within the epithelium covering villi, and the intraepithelial lymphocyte (IEL) count expressed as number of IEL/100 epithelial cells (Ferguson & Murray 1971).

*Statistics.* Crypt cell production rates were compared by covariance analysis, and Student's *t*-test was used for all other results.

## EXPERIMENTS AND RESULTS

Immunization schedules for the three experimental groups of mice are illustrated in Table 1. Separate groups of animals were used for the measurements of lymph node cell migration, and for the measurements of intestinal morphology. Further animals were used as positive controls, immunized systemically with OVA in Freund's complete adjuvant as described above.

In general, animals remained healthy throughout the experiments. Cyclophosphamide-pretreated animals had no clinical side-effects, and weight gain was similar in all groups.

**Table 1.** Treatment of the three experimental groups of mice used in experiments on cyclophosphamide pretreatment and oral immunization

| Day | Cyclophosphamide alone | Ovalbumin alone | Cyclophosphamide/ovalbumin |
|---|---|---|---|
| 2 | 100 mg/kg CY | — | 100 mg/kg CY |
| 1 | — | 2·0 mg OVA | 2 mg OVA |
| 28–37 | — | 0·1 mg OVA | 0·1 mg OVA |
| 37 | Kill | Kill | Kill |

*Serum antibodies*

*Parenteral immunization.* After primary immunization with OVA/FCA and a secondary challenge with OVA, all mice developed high titres of antibody in the haemagglutination assay used ($\log_{10}$ Ab titre $= 4{\cdot}39 \pm 0{\cdot}29$).

*Oral immunization.* None of the orally immunized mice had detectable serum haemagglutinating antibodies to OVA.

*Cell migration studies*

*Parenteral immunization.* As shown in Table 2, lymphoid cells from draining lymph nodes of parenterally immunized animals showed significantly reduced migration in the presence of antigen, the migration index being 0·70.

*Oral immunization.* Table 2 also shows results for cell migration studies using mesenteric lymph nodes of the three experimental groups of mice. There was no significant migration inhibition in the groups with ovalbumin alone or cyclophosphamide alone. However, mesenteric lymph node cells from cyclophosphamide-pretreated, ovalbumin-fed mice had a migration index of 0·63 when cultured in the presence of OVA.

*Intradermal skin testing*

*Parenteral immunization.* Four mice which had received OVA in FCA 21 days previously were tested intradermally with 100 $\mu$g OVA. Skinfold thickness was considerably increased 24 hr later (Table 3) and this increase persisted up to 48 hr (results not shown), confirming this skin test as a measure of delayed hypersensitivity.

*Oral immunization.* Similar skin testing was carried out in two batches of orally immunized mice, 21 and 39 days after immunization with OVA. As illustrated in Table 3, no significant increase in skinfold thickness was elicited in any of the orally immunized groups. Thus, although there was migration inhibition in the presence of ovalbumin in mesenteric lymph node cells of OVA/cyclophosphamide animals, there was no evidence of systemic delayed hypersensitivity to OVA in orally immunized animals.

*Intestinal morphology and epithelial cell kinetics*

Measurements of CCPR and IEL counts were made after 10 days of OVA feeding of the three experimental groups, since these two features appear to be indirect measurements of the presence of a mucosal cell-mediated immune reaction. In addition, a group of untreated, non-immunized control mice was also included in this part of the experiment.

*Histology.* Conventional histological sections showed no abnormality of the mucosa in any of the groups.

*Microdissection and cell kinetic measurements.* The accurate microdissection technique revealed significant although minor differences in intestinal architecture between the three groups. These are summarized in Table 4. Height of villi, depth of crypts and crypt cell production rate in the groups

**Table 2.** Migration indices (mean ± 1 s.d.) of lymph node cells when cultured in presence of 0·1 mg/ml OVA

| Immunized group | Cell source | Migration index* | *P* |
|---|---|---|---|
| OVA/FCA | Pop. LN/Ax LN | 0·70 ± 0·10 | < 0·02 |
| OVA alone | Mesenteric LN | 1·00 ± 0·06 | n.s. |
| OVA/CY | Mesenteric LN | 0·63 ± 0·18 | < 0·005 |
| CY alone | Mesenteric LN | 1·12 ± 0·13 | n.s. |

* For calculation of migration index see Materials and Methods section. Cells from three animals were combined for each test. The values given are the pooled results from four separate experiments. For OVA/FCA and OVA/CY groups, there was significant migration inhibition in all tests.

**Table 3.** Increase in skin thickness (mean ± 1 standard deviation) 24 hr after intradermal injection of antigen or saline in mice orally immunized with ovalbumin

| Experimental group | $n$ | No. days after 1st immunization | Increase in skin thickness (mm) after injection of: 0 saline | 100 μg OVA | $P$ |
|---|---|---|---|---|---|
| 0·1 mg OVA in FCA | 4 | 21 | 0·01 ± 0·05* | 0·79 ± 0·14 | < 0·005 |
| 2 mg OVA orally | 4 | 21 | | 0·10 ± 0·06 | n.s. |
| 2 mg OVA orally + CY pretreatment | 4 | 21 | | 0·06 ± 0·08 | n.s. |
| 100 mg/kg CY | 4 | 21 | | 0·07 ± 0·01 | n.s. |
| 2 mg OVA orally + 0·1 mg OVA daily | 4 | 39 | 0·04 ± 0·04* | 0·00 ± 0·06 | n.s. |
| 2 mg OVA orally + 0·1 mg OVA daily + CY pretreatment | 4 | 39 | | 0·00 ± 0·05 | n.s. |
| 100 mg/kg CY | 4 | 39 | | 0·08 ± 0·09 | n.s. |

* Mean of results from each group combined.

**Table 4.** Sizes of intestinal villi and crypts (mean ± 1 standard deviation) and crypt cell production rate in mice orally immunized with OVA with or without cyclophosphamide pretreatment

| Group | $n$ | Villus height (μm) | Crypt depth (μm) | CCPR (per crypt per hr) |
|---|---|---|---|---|
| Controls | 8 | 656·7 ± 82·3 | 118·7 ± 5·4 | 6·8 |
| OVA alone | 8 | 638·8 ± 124·1 | 118·4 ± 7·8 | 8·4 |
| OVA/CY | 8 | 686·5 ± 90·5 | 132·4 ± 4·3* | 12·0† |
| CY alone | 8 | 680·7 ± 61·2 | 119·1 ± 4·7 | 6·6 |

* $P < 0·001$ against other groups.
† $P < 0·05$ against CY alone and controls.

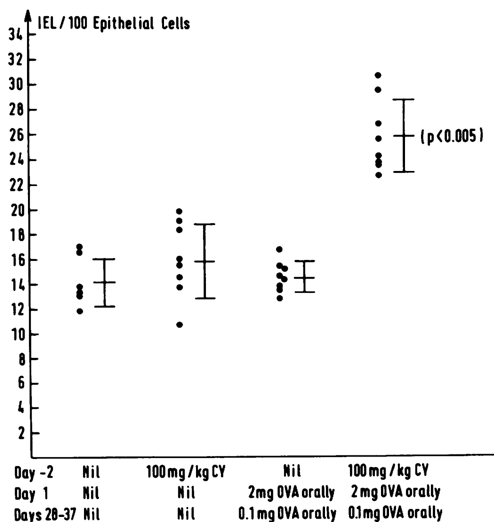


**Fig. 1.** Intraepithelial lymphocyte counts in the jejunum of untreated mice; of animals given cyclophosphamide alone; orally immunized and challenged with ovalbumin; and cyclophosphamide pretreated, orally immunized and challenged with ovalbumin. Bars represent mean ± 1 standard deviation.

with cyclophosphamide alone and ovalbumin alone were similar to those of the untreated controls. However, combined cyclophosphamide pretreatment, oral immunization and 10-day oral challenge resulted in a significant increase in crypt depth (132 compared with 119, 118 and 119 μm) and CCPR (12 compared with 6·8, 6·6 and 8·4). However, there was no significant reduction in villus height.

*Intraepithelial lymphocyte counts.* Fig. 1 illustrates the intraepithelial lymphocyte counts in the controls, and three groups of experimental mice. Values for groups with OVA alone and cyclophosphamide alone are similar to those of controls (mean values being 14·5 and 15·9 IEL/100 epithelial cells, and in controls 14·1) whereas in the cyclophosphamide-pretreated animals, mean IEL count was 25·9.

## DISCUSSION

The experiments described in this paper have shown that, under certain circumstances, oral immunization with a protein antigen induces a local immune response in the GALT which is associated with some alterations in the structure of the intestinal mucosa. The significant inhibition of migration, in the presence of antigen, of cells from the mesenteric lymph nodes of animals pretreated with 100 mg/kg cyclophosphamide before oral immunization and challenge, suggests that cyclophosphamide pretreatment has mainly influenced cell-mediated immune responses. Further support for this isolated effect on CMI is the failure to detect serum antibody after cyclophosphamide pretreatment. A minimal effect on humoral immunity cannot be completely excluded until a more sensitive assay for serum antibody, and measurements of antibodies in secretions, has been used. We chose to use the technique of direct inhibition of migration of mesenteric lymph node (MLN) cells to demonstrate the existence of CMI in the GALT. Preliminary experiments in parenterally immunized animals, who exhibited good delayed hypersensitivity to OVA as measured by skin testing but had poor antibody responses, had shown migration inhibition to be a simple and reproducible *in vitro* test. Further support for the correlation of *in vitro* cell migration inhibition with *in vivo* delayed hypersensitivity is provided by recent work on the correlation of migration inhibition in the presence of purified protein derivative with tuberculin reactivity in humans (Mazuran *et al.*, 1979). There is evidence that the similar phenomenon in mice is T cell-mediated (Castes, Borderie & Orbach-Arbouys, 1978).

Since there is a substantial body of work on the effects of cyclophosphamide on systemic immunity, the enhanced local immune reaction in the gut-associated lymphoid tissues found in our experiments is likely to be the result of a specific immunological effect of cyclophosphamide in releasing CMI responses from suppressor control. However, two other possible actions of cyclophosphamide must also be considered. The small intestinal epithelium is one of the principal target organs for the cytotoxicity of many drugs including cyclophosphamide. Increased permeability, or reduced enzyme content of cells of the intestinal mucosal barrier will alter the capacity of the gastrointestinal tract to handle antigen, and an enhanced CMI response could theoretically result from absorption of an unusually large amount of intact antigen. However, the maximal effects of this dose of cyclophosphamide on the intestinal epithelium occur at 12–24 hr after administration (Ecknauer, 1976; Ecknauer & Lohrs, 1976) and ovalbumin was given to our experimental animals at 48 hr. Another possible effect of cyclophosphamide would be by its action on the antigen-handling capacity of the lymphoid tissues of the gut, for example the Peyer's patches, and their specialized surface epithelium. High doses of cyclophosphamide have been shown, in chickens, to decrease the uptake of protein by the bursal epithelium (Sachs, Beezhold & Van Allen, 1979), but although there have been no similar studies in mammals it seems unlikely that this effect of cyclophosphamide is responsible for the findings in our experiments.

Support for the hypothesis that cyclophosphamide pretreatment reverses GALT suppression comes from the work on systemic delayed hypersensitivity, where it has been found that specific doses of cyclophosphamide, if given 2 or 3 days before antigen, enhance delayed hypersensitivity (Turk, Parker & Poulter, 1972; Askenase *et al.*, 1975; Gill & Liew, 1978). The concept has arisen that there is normally a considerable degree of inhibitory control operating on the induction phase of systemic delayed hypersensitivity responses (Schwartz, Askenase & Gershon, 1978). The source of

this suppression may vary with the system used, and both suppressor B cells (Turk & Poulter, 1972) and suppressor T cells (Rollinghoff *et al.*, 1977; Attallah, Ahmed & Sell, 1979) have been implicated. We cannot draw any conclusions from the work described in this paper as to the nature of the postulated suppressor cells involved, but it is probably significant that suppressor T cells for antibody production have been detected in Peyer's patches of orally immunized animals (Ngan & Kind, 1978; Mattingly & Waksman, 1978) while a similar system appears to exist for cell-mediated immune responses following oral immunization (Miller & Hanson, 1979).

Finally, further support for our hypothesis is provided by an earlier report that orally induced tolerance to a contact sensitizing agent could be reversed by cyclophosphamide pretreatment (Polak, Geleick & Turk, 1975). However, it will be necessary to conduct parallel studies of induction of tolerance to oral antigens as well as antigen transport across the gut epithelium, fully to elucidate the observed effect of cyclophosphamide on local cell-mediated immunity in the gastrointestinal tract.

As stated earlier, our previous work on allograft rejection and graft-versus-host reaction had identified the CCPR and IEL count as the most reliable indices of mucosal CMI (MacDonald & Ferguson, 1976, 1977; Mowat & Ferguson, 1981). In addition, increased CCPR precedes other morphological changes which occur in the intestinal mucosa. Other workers have recently shown that the accumulation of T cells in intestinal mucosa and epithelium is an early feature of GvHR (Guy-Grand, Griscelli & Vassalli, 1978). These two parameters are therefore sensitive if not necessarily specific indicators of a mucosal CMI reaction and were used in the present study to investigate the effects on the intestinal mucosa of manipulation of immune responses to oral antigen. Using these indices, we have shown that when an enhanced CMI response is induced in the GALT by cyclophosphamide pretreatment, significant alterations in small intestinal morphology and epithelial cell kinetics may result. In particular, the increase in crypt mitosis and the IEL count concur with our previous observations on allograft-type reactions in the small intestine. Further support for the concept that immune responses to fed antigen may damage the intestinal mucosa under certain circumstances is provided by a pilot study in which we have immunized and challenged animals with oxazolone after cyclophosphamide pretreatment, and found similar features in the mucosa to those described above (Mowat, unpublished observations).

While the minimal changes in the intestinal mucosa shown in these experiments do not in any way reproduce the villous atrophy and malabsorption as seen in coeliac disease, these changes are consistent with our hypothesis that increased crypt mitosis is one end of the spectrum of damage which can occur in the small intestine as a result of immune responses to dietary antigen (Ferguson & MacDonald, 1977). We now intend to try to produce more severe changes in intestinal morphology by challenging animals with larger doses of antigen and by increasing the time of contact between antigen and gut mucosa by introducing antigen into Thiry–Vella loops.

There is a growing body of evidence that mucosal T cells represent a separate pool of lymphocytes from the systemic T cell system and have a unique origin, route of migration and function (Guy-Grand, Griscelli & Vassalli, 1974; Cahill *et al.*, 1977; Guy-Grand *et al.*, 1978). A separate pool of mucosal B cells has also been defined (Craig & Cebra 1971; Guy-Grand *et al.*, 1974; Rudzik, Perey & Bienenstock, 1975; Befus, O'Neill & Bienenstock, 1978). In theory, activation of the GALT with subsequent migration and differentiation of gut-associated lymphocytes need not involve activation of the systemic immune response. Our findings of enhanced CMI responses in the GALT and mucosa in the absence of systemic delayed hypersensitivity and antibody responses, is further evidence to support the segregation of mucosal and systemic T cell populations.

These experiments may have clinical relevance since they confirm, for a dietary protein antigen, the effects of mucosal CMI which we previously reported in allograft-type reactions. We would postulate that induction of this 'abnormal' immune response to an oral antigen is secondary to a net deficiency of GALT suppressor cell activity which has been revealed by the use of cyclophosphamide. It is possible that similar defects in suppression may occur during early life, when the body encounters many new antigens via the small intestine, and this offers a mechanism for the pathogenesis of the food-allergic diseases which damage the intestine in infancy. The precise effects on the patient would depend on the nature of the suppressor cell defect, whether antigen-specific or not, and whether transient or permanent. Clearly, the suppressor cell system of the GALT,

implicated in these experiments and described by others (Ngan & Kind 1978; Mattingly & Waksman, 1978; Kagnoff, 1978), is of considerable benefit, particularly in infancy when, despite the exposure to large amounts of immunogenic and new antigens, uncontrolled immune responses are rare. The suppressor cell system of the GALT may be one of the most important homeostatic mechanisms during early life.

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