EVIDENCE THAT ACTIVATED MUCOSAL T CELLS PLAY A ROLE IN THE PATHOGENESIS OF ENTEROPATHY IN HUMAN SMALL INTESTINE

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Intestinal damage as a result of allergy and infection is a major cause of morbidity in man and animals (1–3). In intestinal allergy the most commonly seen lesions are small intestinal villous atrophy and crypt cell hyperplasia resulting in malabsorption due to a decreased intestinal absorptive surface and decreased digestive enzyme levels in the epithelial cells. The mechanisms involved in these changes in man are not known, but there is evidence from experimental animal studies that T cells may play an important role in the pathogenesis of the intestinal lesion (4). The enteropathy observed in intestinal graft-versus-host disease and rejection of transplanted intestinal allografts is characterized by a lymphocytic infiltrate, shortening of the villi, lengthening of the crypts and an increased rate of epithelial cell proliferation (5, 6). However, these in vivo models, although useful, do not allow a dissection of the mechanisms involved in the development of enteropathy in human small intestine.

We have recently shown (7) that fetal human small intestine becomes increasingly populated with T cells from ~14 wk of gestation. By 19–22 wk the fetal gut epithelium and lamina propria contain many isolated T cells and the lamina propria contains aggregates of T and B cells that are probably early Peyer's patches (8). Small explants of human fetal small intestine can be maintained in organ culture for several weeks with retention of gut structure and normal epithelial cell function (9). We have thus attempted to directly stimulate mucosal T cells in situ in cultures of human small intestine.

Materials and Methods

Organ Culture of Fetal Human Small Intestine. Small intestine from therapeutically aborted fetuses was placed in a petri dish in serum-free CMRL-1066 medium (Flow Laboratories, Inc., McLean, VA) modified as described by Autrup et al (10). The intestine was cut into segments 2–3 mm in length which were then bisected longitudinally to expose the lumen. The explants were then trimmed into pieces 2–3 mm square. Five pieces of tissue were cultured in 7 ml modified CMRL-1066 medium in 5-cm diameter tissue culture dishes (Sterilin; Scientific Supplies Co. Ltd., Vine Hill, London). The cultures were incubated for 72 h at 37°C in a 95% oxygen, 5% CO atmosphere. At the end of the culture period the five explants in each dish were carefully removed from the

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culture dish and placed on top of one another on a piece of filter paper to absorb excess moisture. The tissues were then snap frozen in liquid nitrogen and stored at −70°C. Frozen sections profiling each of the explants were then cut and stained immunohistochemically using the peroxidase technique as described elsewhere (11). mAbs used for immunohistochemistry were Ki67 (Dako Ltd., High Wycombe, Bucks) which identifies a nuclear antigen in all dividing cells and the anti-T cell antibodies anti-CD3 (Leu-4), CD4 (Leu-3a), CD8 (Leu-2a), and CD25 (anti-Tac) purchased from Becton Dickinson & Co., Mountain View, CA.

Activation of Mucosal T Cells In Situ in Explants of Fetal Small Intestine. Two methods were used. First, PWM (Flow Laboratories, Inc.) was added to the cultures at a concentration of 15 μg/ml, a concentration shown to be optimal in preliminary experiments. Second, anti-CD3 mAb (Serotec Ltd., Kiddington, Oxford) was added to the cultures.

Measurement of $CD25^+$ Cells in the Lamina Propria of Explants of Fetal Small Intestine. Frozen sections of explants of fetal gut were stained immunohistochemically with monoclonal anti-CD25. The number of lamina propria cells staining with this antibody was quantitated by counting the number of stained cells in random fields using the ×40 objective on a Leitz Dialux microscope. At least 10 fields per group were counted.

IL-2 Activity in the Organ Culture Supernatants. IL-2 activity was assayed by a standard method using CTLL-16 cells (12). Briefly, 100 μl of supernatant was diluted twofold in RPMI 1640 with 10% FCS. To each well was added 4,000 CTLL-16 cells. The next day the cells were pulsed with 1 μCi [^{3}H]thymidine and the wells were harvested for liquid scintillation counting 8 h later. Each culture supernatant was measured in duplicate with identical results.

Measurement of Crypt Epithelial Cell Division and Villous Height. Crypt epithelial cell division was measured by counting the number of nuclei staining immunohistochemically with the mAb $Ki67^+$ per crypt in well-oriented sections. Ki67 detects an epitope present on the nuclei of all dividing human cells. The nuclei of cells in G_0 do not stain. At least 10 measurements were made per group. Because the frozen sections of the aggregates of five explants were frequently poorly oriented, all measurements of villous height were done blind. Sections were coded and the heights of the villi that appeared to be sectioned through their length were measured using a calibrated micrometer eyepiece. At least 10 measurements of villous height per group were made.

Statistics. All results are expressed as the mean ± 1 SE. All measurements in any group were first tested to determine if they conformed to a sample from a normally distributed population. If so, differences between groups was determined using analysis of variance. If the values in a group were not normally distributed then groups were compared using the nonparametric Kolmogorov-Smirnov two-group test. All statistics were done using a Microstat statistical program on an Amstrad PC1512 microcomputer.

Results

In Situ Lectin-induced T Cell Activation in the Mucosa of Fetal Human Small Intestine In Vitro. Fetal small intestine from 12 fetuses ranging in age from 14–22 wk gestation was cultured in vitro with PWM (15μg/ml) for 3 d and then examined for the presence of $CD25^+$ cells by immunohistochemistry (Table I). There were few $CD25^+$ cells in the lamina propria of PWM-treated cultures of 14–17-wk-old fetal intestine, but in the specimens aged 18–22 wk these were abundant in the lamina propria. There were no $CD25^+$ cells in control cultures. IL-2 levels in the organ culture supernatants were also measured (representative experiments shown in Fig. 1). Although the levels of activity detected were small, the amount of IL-2 produced after PWM-stimulation also increased with fetal age.

The majority of the cells in the lamina propria of the PWM-stimulated cultures were $CD8^-$. There were numerous $CD4^+$ cells but it was difficult to determine if they were T cells or macrophages since Leu-3a is present on tissue mac-

TABLE I

The Number of CD25$^+$ Cells in the Lamina Propria of Fetal Small Intestinal Organ Cultures of Various Ages after Treatment with PWM

Age of specimen	Exp.	CD25$^+$ cells/× 40 field
wk		
14	1	0
16	1	0
	2	2.3 ± 1.3
	3	2.1 ± 0.9
	4	7.5 ± 1.8
17	1	2.3 ± 0.9
18	1	4.0 ± 0.9
	2	12.5 ± 1.5
20	1	15.9 ± 4.4
21	1	11.2 ± 2.3
	2	19.4 ± 2.5
22	1	21.3 ± 2.6

There were no CD25$^+$ cells in the lamina propria of any of the control organ cultures. All measurements were made after 3 d in culture with PWM (15 μg/ml). CD25$^+$ (anti-Tac) cells in the lamina propria were enumerated by counting the number of cells staining immunohistochemically with monoclonal anti-CD25 (Becton Dickinson & Co.) in random × 40 fields. At least 10 fields per explant were counted. Preliminary experiments showed that 15 μg/ml was the optimal dose of PWM and that the numbers of CD25$^+$ cells in the lamina propria reached peak levels on day 3.

rophages (13). There was no evidence of B cell activation in any of the specimens.

Changes in Mucosal Morphology and Epithelial Cell Proliferation Associated with T Cell Activation. Profound morphologic changes in the explants of small intestine were also seen after the addition of PWM, especially in the 18–22-wk-old tissues. These were best highlighted immunohistochemically using Ki67 to detect dividing cells (Fig 2). Whereas in control cultures there were few epithelial cells in division in the crypts of Lieberkuhn, after the addition of PWM there was a

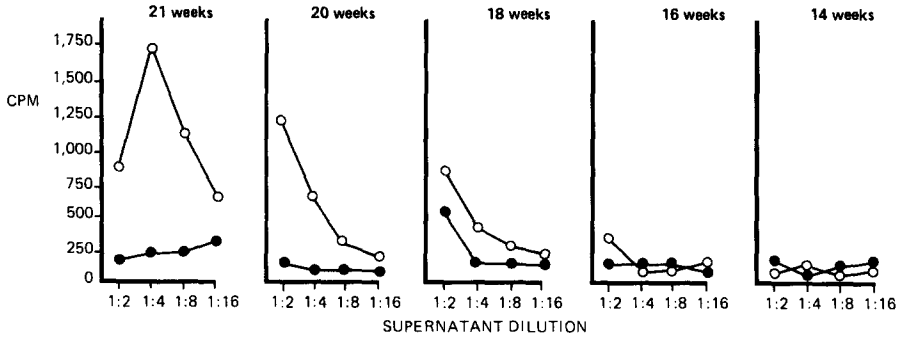

FIGURE 1. IL-2 activity in the culture supernatants of explants (five/dish) of fetal small intestine of different ages. (—○—) Cultures stimulated with 15 μg/ml PWM, (—●—) control cultures. All supernatants were harvested after 2–3 d of culture. CTLL-16 cells cultured in medium alone gave 100–200 cpm. In a 1:2 dilution of MLA-144 supernatant used as a standard, the CTLL-16 gave 20,000 cpm.

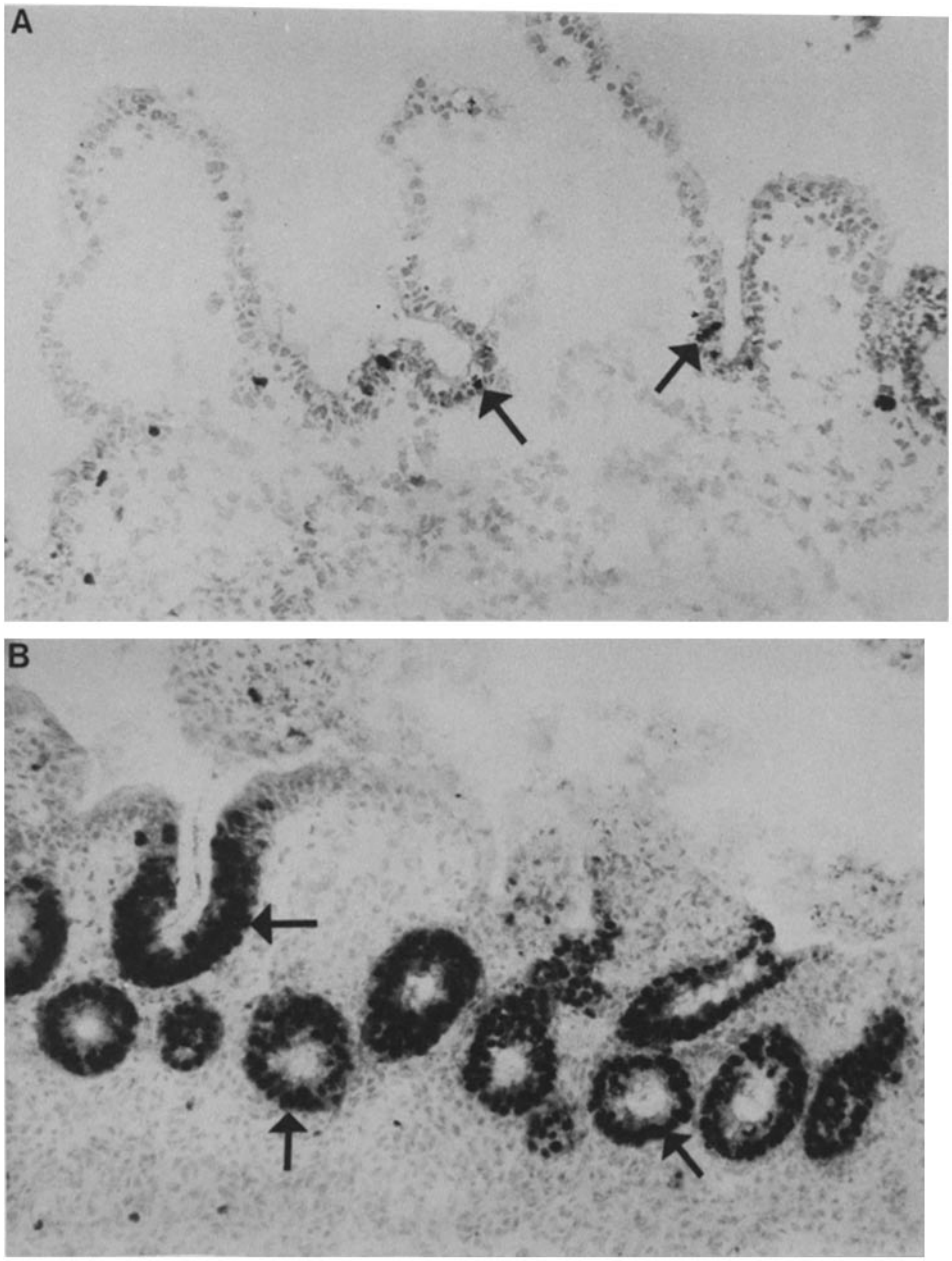

FIGURE 2. The morphologic changes produced by the addition of PWM to in vitro cultures of explants of 18-wk-old fetal small intestine (immunoperoxidase, × 40). Shown are frozen sections of fetal gut cultured for 72 h in medium alone (*A*) or 15 μg/ml PWM (*B*). The sections were stained immunohistochemically with the mAb Ki67 (Dako Ltd.), which stains the nuclei of dividing cells, to highlight the increase in the number of dividing crypt epithelial cells (arrows). In control cultures, the villi are long and the crypts are short, with few $Ki67^+$ cells. In contrast, in cultures to which PWM has been added there is almost total villous atrophy and intense epithelial cell proliferation in the crypts. The oedema in the villous core of the control cultures is due to water absorption by the villous epithelial cells that accumulates in the villi because there is no lymphatic or venous drainage.

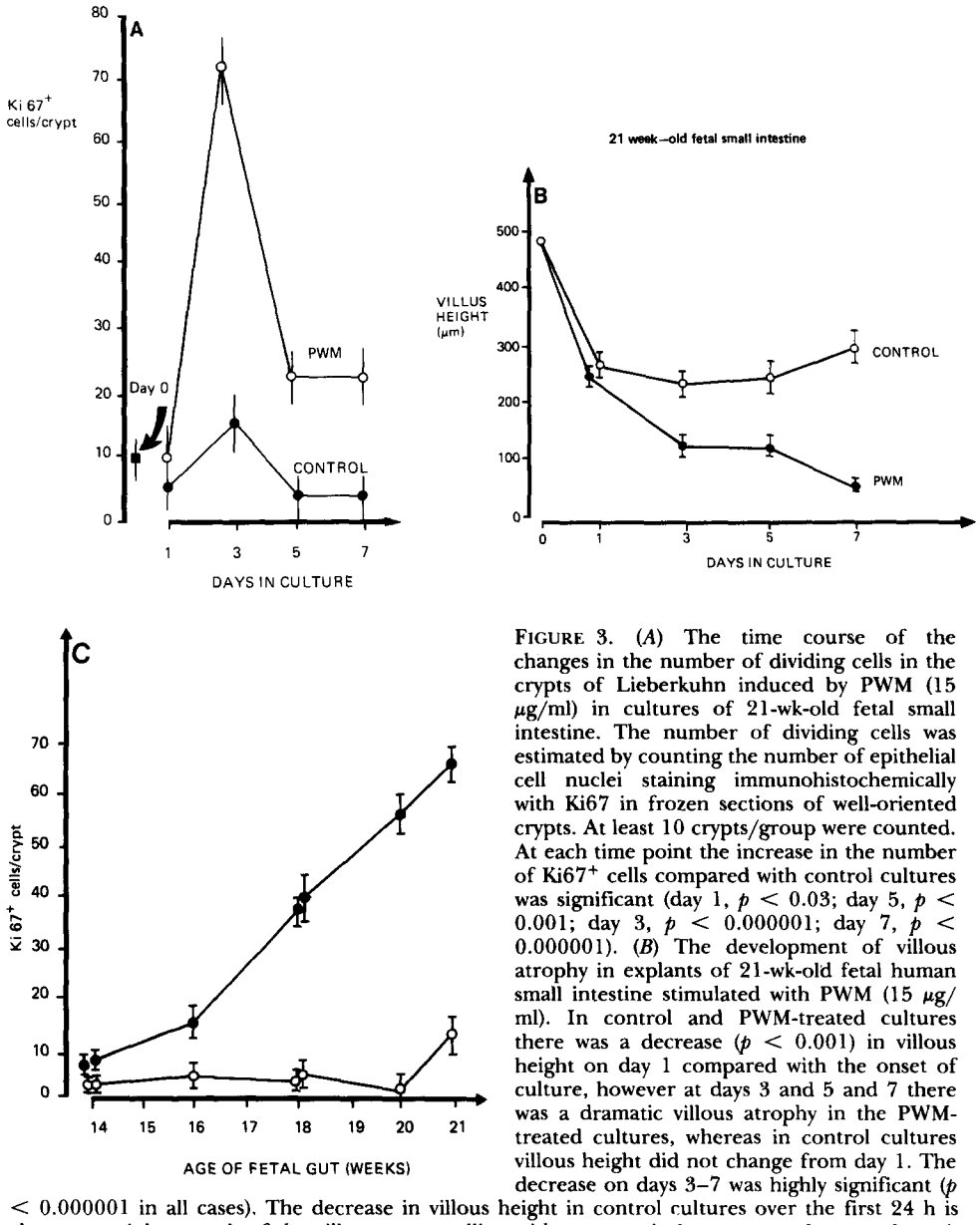

FIGURE 3. (*A*) The time course of the changes in the number of dividing cells in the crypts of Lieberkuhn induced by PWM (15 μg/ml) in cultures of 21-wk-old fetal small intestine. The number of dividing cells was estimated by counting the number of epithelial cell nuclei staining immunohistochemically with Ki67 in frozen sections of well-oriented crypts. At least 10 crypts/group were counted. At each time point the increase in the number of $Ki67^+$ cells compared with control cultures was significant (day 1, $p < 0.03$; day 5, $p < 0.001$; day 3, $p < 0.000001$; day 7, $p < 0.000001$). (*B*) The development of villous atrophy in explants of 21-wk-old fetal human small intestine stimulated with PWM (15 μg/ml). In control and PWM-treated cultures there was a decrease ($p < 0.001$) in villous height on day 1 compared with the onset of culture, however at days 3 and 5 and 7 there was a dramatic villous atrophy in the PWM-treated cultures, whereas in control cultures villous height did not change from day 1. The decrease on days 3–7 was highly significant ($p < 0.000001$ in all cases). The decrease in villous height in control cultures over the first 24 h is almost certainly a result of the villous core swelling with water actively transported across the epithelial cells from the culture medium. (*C*) The relationship between the age of the fetal intestine and the degree of crypt cell hyperplasia induced by PWM (15 μg/ml in all cases). All specimens were examined after 3 d of culture. Two 14-wk-old fetuses were studied, only one of which did the PWM induce an increase in $Ki67^+$ cells ($p < 0.05$). In all the older specimens studied the PWM induced a significant increase in $Ki67^+$ cells (16 wk, $p < 0.05$; both 18-wk-old specimens, $p < 0.002$; 20 wk, $p < 0.003$; 21 wk, $p < 0.008$).

dramatic crypt cell hyperplasia which peaked at 3 d but was still evident after 1 wk in culture (Fig. 3 *A*). The crypt hyperplasia was also associated with villous atrophy (Fig 3 *B*). It was also clear that the extent of the crypt hyperplasia increased with the age of the tissue (Fig. 3 *C*).

TABLE II

Cyclosporin A Inhibits the Development of Crypt Epithelial Cell Hyperplasia and the Appearance of CD25$^+$ cells in PWM-treated Organ Cultures of Fetal Human Small Intestine

	Exp. 1 (16-wk-old gut)		Exp. 2 (20-wk-old gut)	
	Ki67$^+$ cells per crypt	CD25$^+$ cells per field	Ki67$^+$ cells per crypt	CD25$^+$ cells per field
Control	0.7 ± 0.3	0	0.4 ± 0.2	0
PWM (15 μg/ml)	12.0 ± 3.7	7.5 ± 1.8	35.2 ± 2.7	15.8 ± 4.4
Cyclosporin A (15 μg/ml)	0.7 ± 0.5	0	1.6 ± 0.6	0
PWM + Cyclo A	2.3 ± 0.9	0.3 ± 0.2	1.0 ± 0.5	2.2 ± 0.5

Each group contained five fetal small intestinal explants. The tissue was snap frozen on day 3 of culture and the number of dividing cells in the crypts measured in frozen sections as described in Fig 3. CD25$^+$ (anti-Tac) cells in the lamina propria were enumerated by counting the number of cells staining immunohistochemically with monoclonal anti-CD25 (Becton Dickinson & Co.) in random × 40 fields. At least 10 fields per explant were counted. The PWM and Cyclosporin A were added at the onset of the culture. Cyclosporin A (Sandoz) was diluted in culture medium to the appropriate concentration. In each experiment the increase in the number of Ki67$^+$ cells/crypt induced by PWM was significant ($p < 0.001$ for the 20-wk-old gut, $p < 0.0002$ for the 16-wk-old gut), as was the reduction produced by the addition of the Cyclosporin A ($p < 0.001$, for the 20-wk-old gut, $p < 0.004$ for the 16-wk-old gut). Similarly the reduction in the number of CD25$^+$ cells was significant ($p < 0.05$, for the 16-wk-old gut, $p < 0.002$ for the 20-wk-old gut).

Cyclosporin A Inhibits the Development of Crypt Cell Hyperplasia in PWM-treated Fetal Small Intestine. To help exclude the possibility that the changes observed were due to the direct effect of PWM on the epithelial cells we added Cyclosporin A (Table II), a potent inhibitor of T cell activation (14, 15), to some of the PWM-stimulated cultures. The addition of 15 μg/ml Cyclosporin A at the onset of culture almost completely prevented the development of CD25$^+$ cells in the lamina propria and crypt epithelial cell hyperplasia.

T Cell Activation with Anti-CD3 Antibody Results in Crypt Epithelial Cell Hyperplasia. Explants of fetal intestine were also treated with anti-CD3 antibody to directly activate mucosal T cells. This resulted in the appearance of CD25$^+$ cells in the lamina propria of the explants (not shown) and a dose-dependent increase in the number of dividing crypt epithelial cells (Table III).

Discussion

These studies indicate that activation of mucosal T cells in human small intestine in vitro rapidly produces crypt epithelial cell hyperplasia and villous atrophy. The degree of mucosal lymphocyte activation was related to the age of the tissue studied. Noticeably, PWM had virtually no effect on 14-wk-old fetal intestine, which has few T cells, as shown previously (7, 8). As the age of the specimens tested increased and the mucosa contained more T cells at the onset of culture so the PWM induced more CD25$^+$ cells in the lamina propria and the greater the mucosal changes. Cyclosporin A also inhibited both the development of CD25$^+$ cells and tissue damage after the addition of PWM. Finally, anti-CD3

TABLE III
Activation of Mucosal T Cells with Anti-CD3 Antibody Results in Dose-dependent Crypt Epithelial Cell Hyperplasia

Concentration of anti-CD3 antibody	$Ki67^+$ cells per crypt mean ± 1 SE
μg/ml	
—	3.7 ± 1.4
0.1	8.0 ± 2.2
0.5	15.0 ± 2.3
2.5	20.2 ± 2.5

Explants from a 17-wk-old fetus were cultured in anti-CD3 at the indicated concentrations. 3 d later the tissues were snap frozen and stained immunohistochemically with Ki67. The increase in Ki67 cells/crypt induced by 0.5 and 2.5 μg/ml anti-CD3 were highly significant ($p < 0.0001$).

antibody–induced mucosal T cell activation also resulted in a dose-dependent crypt hyperplasia. Taken together, these results provide compelling evidence that a consequence of mucosal T cell activation is crypt epithelial cell hyperplasia and villous atrophy. We consider it unlikely that the PWM-induced effects are due to direct effects on epithelial cells for two reasons. First, in preliminary electron microscopy studies we are unable to demonstrate epithelial cell damage after the addition of PWM. Second, biotin-labeled PWM binds to the epithelial cells of 14-wk-old fetal intestine to the same extent as in older tissues (not shown), thus making it unlikely that the failure of the PWM to induce changes in intestine of this age is due to the absence of PWM receptors.

In this model we used a polyclonal activator or anti-CD3 antibody to stimulate mucosal T cells, but we would envisage that clinical enteropathy occurs when sensitized small intestinal lymphocytes interact with nominal antigen from the gut lumen, such as gluten in the case of celiac disease. One of the advantages of working with sterile explants of fetal small intestine in vitro is that the interpretation of the results is not confused by multiple immunologic events. For example, in the case of a celiac patient challenged with gluten, the patient will almost certainly have high levels of antibody to gluten (16), capable of causing immune complex deposition in the mucosa (17).

The mechanisms by which T cells mediate the enteropathy described here are unknown. The most striking feature to us was the rapid and profound increase in the rate of crypt epithelial division, which may suggest a direct effect of the activated T cells or their products on these cells. PWM induces at least a subset of the lamina propria T cells to secrete lymphokines since we were able to detect IL-2 and IFN-γ (data not shown) in the supernatants of the damaged explants. However, these lymphokines on their own are not responsible for the mucosal changes, since we have tested both recombinant lymphokines at a variety of doses and they have little effect on mucosal morphology (negative data, not shown). Rather, the presence of IL-2 in the organ culture supernatant was only used to show that PWM treatment resulted in functional T cell activation. Further investigation is necessary to determine if any known (or perhaps new) lym-

phokine(s) are capable of influencing the rate of epithelial cell renewal, either directly or via interaction with mesenchymal cells in the lamina propria.

Summary

T cells in explants of human fetal small intestine in organ culture were stimulated in situ with PWM or anti-CD3 antibody to test the hypothesis that activated T cells produce enteropathy in human small intestine. T cell activation was measured by the appearance of $CD25^+$ cells in the lamina propria of the explants and IL-2 production into the organ culture supernatant. We have previously shown (7, 8) that the number of T cells in human fetal gut increased between 14 and 22 wk gestation. Accordingly, after the addition of PWM to cultured explants of fetal intestine the number of $CD25^+$ cells in the lamina propria and the amounts of IL-2 secreted into the organ culture supernatant increased with the age of the explanted tissue. The addition of PWM also produced an age-related enteropathy, most noticeably crypt epithelial cell hyperplasia and villous atrophy, with relatively minor changes in 14–17-wk-old intestine but severe tissue damage in 18–22-wk-old fetal intestine. These enteropathic effects were also produced when mucosal T cells were activated with anti-CD3 mAb. Cyclosporin A completely inhibited the PWM-induced development of $CD25^+$ cells and related tissue damage. These experiments show that activated T cells in human small intestine produce enteropathy. The model provides a new system with which to dissect the mechanisms of T cell–mediated intestinal damage.

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References

1. Ferguson, A. 1976. Coeliac disease and gastrointestinal food allergy. *In* Immunological Aspects of the Liver and Gastrointestinal Tract. Anne Ferguson and R. N. M. McSween, editors. MTP Press Ltd, Lancaster. 153–202.
2. Miller H. R. P. 1984. The protective mucosal response against gastrointestinal nematodes in ruminants and laboratory animals. *Vet. Immunol. Immunopathol.* 6:168.
3. Savilhati, E. 1986. Morphologic changes and immune reactions in the intestine of patients with food allergy. *In* Paediatric Gastroenterology. D. Branski, G. Dinari, P. Rozen, and J. A. Walker-Smith, editors. S. Karger AG, Basel. 55–68.
4. Ferguson, A., and T. T. MacDonald. 1977. Effects of local delayed hypersensitivity in the small intestine. *Ciba Found. Symp.* 46:305–327.
5. MacDonald, T. T., and A. Ferguson. 1976. Hypersensitivity reactions in the small intestine. 2. Effects of allograft rejection on mucosal architecture and lymphoid cell infiltrate. *Gut.* 17:81.
6. MacDonald, T. T., and A. Ferguson. 1977. Hypersensitivity reactions in the small intestine. 3. The effects of allograft rejection and graft-versus-host disease on epithelial cell kinetics. *Cell Tissue Kinet.* 10:301.
7. Spencer, J., S. B. Dillon, P. G. Isaacson, and T. T. MacDonald. 1986. T cell subclasses in fetal human ileum. *Clin. Exp. Immunol.* 65: 553.

8. Spencer, J., T. T. MacDonald, T. Finn, and P. G. Isaacson. 1986. The development of gut associated lymphoid tissue in the terminal ileum of fetal human intestine. *Clin. Exp. Immunol.* 64:536.
9. Menard, D., and P. Arsenault. 1985. Explant culture of human fetal small intestine. *Gastroenterology.* 88:691.
10. Autrup., H., L. A. Barrett, F. E. Jackson, et al. 1978. Explant culture of human colon. *Gastroenterology.* 74:1248.
11. Isaacson, P. G., and D. H. Wright. Immunocytochemistry of lymphoreticular tumours. *In* Immunocytochemistry. Practical Applications in Pathology and Biology. J. Polak and S. van Noorden, editors. John Wright and Sons, Bristol. 249.
12. Baker, P. S., S. Gillis, and K. A. Smith. 1979. Monoclonal cytolytic T cell lines. *J. Exp. Med.* 149:273.
13. Wood, G. S., W. L. Warner, and R. A. Warnke. 1983. Anti-Leu 3/T4 antibodies react with cells of monocyte/macrophage and Langerhans lineage. *J. Immunol.* 131:212.
14. Granelli-Piperno, A., K. Inaba, and R. M. Steinman. 1984. Stimulation of lymphokine release from T lymphoblasts. Requirement for mRNA synthesis and inhibition by Cyclosporin A. *J. Exp. Med.* 160:1792.
15. Elliot, J. F., Y. Lin, S. B. Mizel, et al. 1984. Induction of Interleukin-2 messenger RNA inhibited by Cyclosporin A. *Science (Wash. DC).* 226:1439.
16. Ferguson, A., and F. Carswell. 1972. Precipitins to dietary proteins in serum and upper intestinal secretions of coeliac children. *Br. Med. J.* 1:75.
17. Shiner, M., and J. Ballard. 1972. Antigen-antibody reactions in jejunal mucosa in childhood coeliac disease after gluten challenge. *Lancet.* i:1202.