THE EFFECT OF IMMUNE CELLS AND THEIR PRODUCTS ON THE GROWTH AND DIFFERENTIATION OF SMALL INTESTINAL EPITHELIAL CELLS *IN VITRO*

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SUMMARY

Crypt hyperplasia in the small intestine is one of the early features of a group of enteropathies which include coeliac disease and it is thought that this phenomenon may be caused by cytokines produced during a DTH response. The work in this thesis was aimed at developing *in vitro* models to test the hypothesis that cytokines act directly on dividing crypt epithelial cells to produce crypt hyperplasia.

The RIE-1 cell line, derived from rat small intestine, appears to represent immature crypt-like cells and thus I considered it to be a suitable model for my studies.

Initially, I investigated the effects of T lymphocytes on RIE-1 cell growth. ConA stimulated MLN cells and their supernatants decreased the growth of the RIE-1 cells, and the cytostatic effect of the latter was partially reversed by an anti-IFNγ monoclonal antibody.

Macrophages also had a cytostatic effect on RIE-1 cells, which could be further enhanced by stimulation of the macrophages with IFNγ. Inhibiting prostaglandin and NO production had no major effects on the growth inhibition caused by macrophages. LPS stimulated macrophages produced TNFα, and the cytostatic effects of these cells were partly inhibited by antibody against this cytokine. Macrophage supernatants were also cytostatic to RIE-1 cells and, in general, the effects of supernatants from macrophages activated by the different agents correlated with those of macrophages themselves.

To examine the role of particular mediators, I next examined the effects of individual cytokines on the growth of RIE-1 cells. IFNγ, TGFβ and TNFα were cytostatic to exponentially growing cultures of RIE-1 cells, but only IFNγ was cytostatic to confluent cultures of cells. IL1 and TNFα had a proliferative effect on confluent cultures of RIE-1 cells. A number of other cytokines had no effect.

In an attempt to produce a more accurate model, I co-cultured RIE-1 cells with a fibroblast monolayer. RIE-1 cells in co-culture formed structures several layers thick and a small proportion took on a columnar appearance and had increased numbers of cytoplasmic organelles. However, there were no other obvious features of differentiation and as I was unable to measure the growth of RIE-1 cells quantitatively in this model, I decided it was unsuitable for further use.

Finally, I attempted to establish primary cultures of small intestinal epithelial cells. Intestinal epithelial cells isolated from neonatal rats grew in culture and were shown to express digestive enzymes and cytokeratins. EM also showed the presence of columnar epithelial cells with tight junctions and desmosomes and a brush border of microvilli. However, the cultures were contaminated by the presence of non-epithelial cell types making them unsuitable for use in my studies.

The overall conclusion of this thesis is that the RIE-1 cell line provides a useful model to study the effects of mediators on the growth of small intestinal epithelial crypt cells *in vitro* but is unsuitable for differentiation studies. Cytokines derived from T lymphocytes and macrophages did have effects on RIE-1 cells, but these were mainly cytostatic, suggesting that cytokines may not produce crypt hyperplasia via direct action on crypt stem cells in enteropathy as has been hypothesised. If such a stimulatory effect does occur *in vivo*, I would propose that this occurs via an indirect effect of cytokines on for example mesenchymal cells. One cytokine which may explain this activity is IL1, which is produced by fibroblasts and was stimulatory to RIE-1 cells. My results indicate that it may contribute to hyperplasia by recruiting non-dividing crypt epithelial cells back into the cell cycle. Finally, my results underline the difficulty in examining enterocyte growth and

differentiation *in vitro* and indicate that there is as yet no perfect model for this important phenomenon.

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Declaration

These studies represent original work carried out by the author, and have not been submitted in any form to any other University. Where use has been made of material provided by others, due acknowledgement has been made.

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Part of this thesis has been included in the following publication:-

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Abbreviations used in the text

B lymphocyte	bone marrow derived lymphocyte
BM	bone marrow
BSA	bovine serum albumin
CD3	T cell marker
CD4	T helper cell marker
CD8	cytotoxic T cell marker
CMI	cell mediated immunity
CO_2	carbon dioxide
ConA	concanavalin A
cpm	counts per minute
^{51}Cr	sodium chromate
CTL	cytotoxic T lymphocyte
DAB	diamino benzidine
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DTH	delayed type hypersensitivity
EGF	epidermal growth factor
EHS	Engel Holm Sarcoma
ELIS	enzyme linked immunosorbant
F_1	first generation
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FGF	fibroblast growth factor

GvHR	graft-versus-host reaction
HBSS	Hank's basic salt solution
HCl	hydrochloric acid
^{3}H-TdR	tritiated thymidine
IEL	intraepithelial lymphocyte
IFNα/β	interferon alpha/beta
IFNγ	interferon gamma
Ig	immunoglobulin
IL1	interleukin 1
IL2	interleukin 2
IL3	interleukin 3
IL4	interleukin 4
IL6	interleukin 6
LPS	lipopolysaccharide
L-NMMA	L-N monomethyl arginine
MHC	major histocompatability complex
MLN	mesenteric lymph node
MMC	mucosal mast cell
mRNA	messenger ribonucleic acid
MTT	(3-4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCS	newborn calf serum
NK	natural killer cell

NO	nitric oxide
p	probability
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
s.e.	standard error
SNAP	s-nitroso-acetyl penicillamine
T lymphocyte	thymus derived lymphocyte
TGFα	transforming growth factor alpha
TGFβ	transforming growth factor beta
TNFα	tumour necrosis factor alpha
VIP	vasoactive intestinal peptide

Length

cm	centimetre
μm	micrometre
nm	nanometre

Volume

l	litre
ml	millilitre
μl	microlitre

Weight

g	gram
mg	milligram
μg	microgram

ng	nanogram

Time

hr	hour
min	minute
sec	second

Concentration

M	molar
mM	millimolar
nM	nanomolar

Symbols

<	less than
>	greater than
=	equal to
/	per

CHAPTER 1

INTRODUCTION

The Structure of the Small Intestine

The function of the small intestine is nutrient absorption. This is carried out by a single layer of columnar epithelial cells which cover the surface of the small intestine and allow selective passage of nutrients, electrolytes and water from the lumen of the gut into the body. The structure of the small intestine reflects its function. Small finger-like projections called villi, each of which is associated with one or more pocket-like crypts, cover the surface of the small intestine and increase the surface area for absorption. The monolayer of epithelial cells is constantly being renewed. Stem cells in the base of the crypt divide continuously and the immature cells produced move up the crypts, differentiating until they become mature absorptive cells on the villus. The villus absorptive cells are polarised columnar cells whose apical surface has a brush border of microvilli, containing the digestive enzymes such as aminopeptidase, sucrase and lactase (which are not present in immature crypt cells) and are joined to each other by tight junctions and desmosomes. On reaching the tip of the villus, the epithelial cells are extruded into the lumen. Under normal conditions, a steady state is maintained, with the production of epithelial cells in the crypt balancing the loss of epithelial cells at the tip of the villus.

The gut is also a constant source of foreign antigens. Although most of these are harmless such as food antigens, some are potentially harmful and so the gut requires effective immune protection. The small intestine contains large numbers of cells of the immune system which reside both in the epithelium and deeper layers of the intestine. These cells include T lymphocytes, B lymphocytes, NK cells, macrophages, mast cells and eosinophils and the intestine has the

capacity to mount potent local cell mediated immune responses. The principal role of a mucosal cell mediated immune response is to produce protective immunity against pathogens, but it can also cause hypersensitivity reactions which may result in intestinal pathology. This may occur when an inappropriate immune response is mounted against a harmless antigen, or when an initially specific response causes non-specific tissue damage.

Enteropathy due to Intestinal Cell Mediated Immunity

A number of human enteropathies are associated with local cell mediated hypersensitivity responses of this type, including coeliac disease, cow's milk protein intolerance, and certain parasite infections (Mowat, 1984). These enteropathies are characterised by diarrhoea and malabsorption and are important causes of morbidity, especially in children. Similar pathology is found in graft versus host disease after allogeneic bone marrow transplant, and during rejection of small bowel allografts (Ferguson and Parrott, 1972, 1973, MacDonald and Ferguson, 1976) and there may also be an element of cell mediated immune reaction in Crohn's disease (Choy et al., 1990). As current treatments are often unsatisfactory, elucidation of the mechanisms underlying these enteropathies could allow specific preventative therapy to be developed.

The pathology of these small intestinal disorders is characterised by crypt hyperplasia, villus atrophy and inflammation, which is associated with increases in the numbers of intraepithelial lymphocytes, mucosal mast cells and the expression of MHC class II molecules by the epithelial cells (Marsh, 1988, Arnaud-Battandier et al., 1986). This pattern is typified best by coeliac disease, which is

caused by intolerance to gluten, a component of wheat. In established disease, intense crypt hyperplasia is accompanied by a flat mucosa in which the villi have disappeared and there is a decreased absolute number of intraepithelial lymphocytes (Marsh, 1988). However, evidence suggests that this pattern represents the severe end of a spectrum of progressing damage, which begins with an increase in the absolute number of IEL, especially in the crypts, followed by crypt hypertrophy and hyperplasia, but normal villi. Villus atrophy only occurs at the later, more severe stage of enteropathy. This milder stage has been seen in some relatives of patients with coeliac disease, and such "latent" coeliac disease has been confirmed by detailed studies of the response of treated coeliac patients to challenge with gluten (Marsh, 1988). Administering gluten orally to coeliac patients initially causes crypt hyperplasia and increases in intraepithelial lymphocytes, although at this point, the villi remain normal. However, later on there is a considerable decrease in the villus height. Thus, crypt hyperplasia seems to be the primary event in this enteropathy and is not merely a compensatory response to a decrease in the villus cell numbers.

Experimental Models of Enteropathy

That crypt hyperplasia is the primary event in enteropathy has been confirmed in a number of animal models including allograft rejection, parasite infections and intestinal graft versus host reaction.

Transplantation of allografts of foetal small intestine under the kidney capsule of mice produces a cell mediated immune response whose earliest sign is the infiltration of the lamina propria by lymphocytes, followed by crypt hyperplasia and an increase in the

density of intraepithelial lymphocytes (Ferguson and Parrot, 1972, 1973 MacDonald and Ferguson, 1976). This rejection has been shown to be thymus dependent (Ferguson and Parrott, 1973). Villus atrophy does not appear until just before complete rejection occurs, at which time the crypts remain significantly hypertrophied.

Parasite infections with Giardia and Hexamita have been shown to cause a similar pathology with increases in intraepithelial lymphocytes, and also crypt hyperplasia in the small intestine of infected mice (MacDonald and Ferguson, 1978). Nematode infections such as Trichinella spiralis and Nippostrongylus also produce severe intestinal damage which includes crypt hyperplasia and villus atrophy and which is reduced in athymic rodents. In addition, the enteropathy of T. spiralis can be prevented by Cyclosporin A, supporting the role of the cell mediated immune response in the pathology (Garside et al., 1992).

The intestinal phase of GvHR, which occurs in mice injected with allogeneic T lymphocytes, provides the most detailed evidence that a local cell mediated immune response can produce a progressive enteropathy which evolves from proliferation to destruction. There are two principal models of intestinal GvHR. The first is an entirely proliferative disorder characterised by increases in crypt cell production rate, crypt length, intraepithelial lymphocytes and mucosal mast cells (Mowat and Ferguson, 1982). Villus atrophy does not occur at any time in these animals. The second model causes a more severe form of mucosal pathology, in which there is an initial proliferative phase of crypt hyperplasia and increases in intraepithelial lymphocytes (Mowat et al., 1988, Mowat and Felstein, 1990). As the disease progresses, destructive pathology occurs, with marked villus

shortening. As crypt hyperplasia always precedes villus atrophy then it possible that villus atrophy may be a consequence of the crypt hyperplasia.

Mechanisms of Experimental Enteropathy

The cell mediated immune response is a complex phenomenon involving different populations of T lymphocytes and other cell types, but the work on intestinal GvHR provides several pieces of evidence indicating that soluble mediators produced by a DTH reaction may be important. Intestinal GvHR is caused by class II restricted $CD8^-$ T cells (Piguet, 1985, Mowat et al., 1986) and there is no correlation with anti-host CTL activity suggesting that the pathology is not due to specific anti-host cytotoxicity (Borland et al., 1983). The epithelial cell damage occurs as a bystander phenomenon secondary to recognition of BM derived cells, as parental strain mice made chimeric for F_1 bone marrow cells develop GvHR despite the fact that the intestinal epithelium of these animals remains syngeneic to parental donor BM cells (Mowat, 1986). In addition, grafts of intestinal tissue which are syngeneic to the parental donor cells, develop pathology similar to that seen in the host intestine when a GvHR is induced in F_1 hosts (Mowat, 1981). Thus, the pathology is not due to donor T cells specifically attacking enterocytes and it has been hypothesised that cytokines produced by the immune cells may be responsible.

There is also activation of several non specific immune cells in intestinal GvHR such as mucosal mast cells. However, MMC do not contribute to the pathology, as mast cell deficient mice develop a normal intestinal GvHR (Newlands et al., 1990). NK cells are activated in GvHR and, although they are not required to induce GvHR, depletion

of NK cells prevents the enteropathy in GvHR (Mowat and Felstein, 1987).

A similar pattern of evolving enteropathy occurs when T lymphocytes are activated in explants of human foetal intestine *in vitro* and provides additional evidence that T cell derived cytokines are important in enteropathy (MacDonald and Spencer, 1988). Addition of pokeweed mitogen to organ cultures causes an increase in activated $CD4^+$ T cells in the lamina propria, which is accompanied by crypt hyperplasia and an increase in IEL. Villus atrophy also occurs, but this appears later than the changes in the crypt. The pathology is prevented by inhibiting T cells with cyclosporin A or anti-CD3 antibody. IL2 and $IFN\gamma$ are produced by the lamina propria $CD4^+$ T cells, although addition of these cytokines to the explants has no effect on mucosal morphology. Thus, cytokines produced during a delayed type hypersensitivity response appear to cause crypt hyperplasia, which is later followed by villus atrophy.

This is supported by both *in vivo* and *in vitro* work, where enteropathy caused by T. spiralis can be prevented by cyclosporin A, whose major function is to block the production of T lymphocyte cytokines. Again this suggests that cytokines play an important role in the development of enteropathy (Garside et al., 1992).

There is also evidence that cytokines may be involved in clinical enteropathy. Increased numbers of T lymphocytes expressing IL2 receptors are found in the lamina propria of patients with Crohn's disease (Choy et al., 1990) and, using ELI-spot techniques, it has been shown that patients with inflammatory bowel disease have an increased number of $TNF\alpha$ secreting cells in the small intestine (MacDonald et al., 1990). Although the latter work could not identify

IFNγ secreting cells in either control or diseased biopsies, more sensitive techniques have recently shown the presence of IFNγ producing cells in inflammatory bowel disease mucosa (Breese et al., 1993).

Together, these models show that activated $CD4^+$ T cells can cause crypt cell hyperplasia and villus atrophy both *in vivo* and *in vitro* and indicate that villus atrophy may be a consequence of alterations in crypt cell turnover. Specific cytotoxicity to epithelial cells does not appear to cause the pathology and it seems that the pathology is due to a DTH response with release of cytokines. It has been hypothesised that dividing crypt cells may be the primary target of this response with an increased rate of turnover resulting in crypt hyperplasia. Intense crypt hyperplasia may then result in a hyperdynamic, unstable mucosa and ultimately cause villus atrophy.

Immune Cells Involved in GvHR and Related Enteropathies

In order to understand the mechanisms leading to enteropathy, it is important to identify the immune cells and their products which cause the pathology. Many immune cells are present in the normal intestinal mucosa including T lymphocytes, B lymphocytes, macrophages, dendritic cells, eosinophils and mast cells (Parrot, 1987). During an intestinal GvHR, a cell mediated immune response takes place, thus many of these different types of immune cells will be activated and in increased numbers in the small intestine. In particular, T lymphocytes, macrophages and NK cells will play an active role in the response and these cells produce a wide range of cytokines which may act directly on epithelial cells or act indirectly via other cells and thus cause the proliferative enteropathy. Thus, the

immune cells which I have focused on in this thesis are T lymphocytes, macrophages, and NK cells.

I have already discussed the evidence that activated T lymphocytes can cause enteropathy. However, macrophages, are also abundant in the gut, and play an important role in cell mediated immune responses and inflammation, via their phagocytic activity, their ability to produce a wide range of cytokines and by their role in antigen presentation (Nathan, 1987). They also increase in numbers in the gut during Crohn's disease (Selby et al 1983) and increased numbers of TNFα secreting cells, presumably macrophages, have been demonstrated in children with Crohn's disease (MacDonald et al., 1990). During an intestinal GvHR in mice, macrophages have been shown to be primed to produce TNFα (Nestel et al., 1992).

NK cells are large granular lymphocytes which upon activation with IL-2, IFNγ or IFNα/β have non-specific, non-MHC-restricted cytolytic activity against tumour cells and also certain normal cells. Upon activation they also secrete a number of cytokines including IL2 and IFNγ (Handa et al., 1983, Kasahara et al., 1983). They increase in numbers during intestinal GvHR and depletion of NK cells in a GvHR results in a less severe enteropathy with less splenomegaly and no crypt hyperplasia (Mowat and Felstein, 1987). NK cells could therefore contribute to enteropathy via cytokine secretion or via direct cytotoxicity against epithelial cells.

The Role of Cytokines in Immunologically Mediated Enteropathy

Recent studies in mice have attempted to show directly a role for cytokines in experimental enteropathy and to identify some of the mediators involved. Increased levels of IL1, IFNγ, TNFα, IFNβ and IL6

have been shown in several models of intestinal GvHR (Smith et al., 1991, Cleveland et al.,1987) and a number of these mediators have been implicated in enteropathy.

In vivo depletion of IFNγ prevents the crypt hyperplasia, villus atrophy and increases in IEL numbers normally found in mice with GvHR (Mowat, 1989). Administering normal mice with IFNγ produces a mild villus shortening (Garside et al., 1993), while the levels of IFNγ found in GvHR correspond with the proliferative stage of GvHR (Garside et al., 1994). These results suggest that this cytokine is important in early stages of enteropathy and may cause crypt hyperplasia. This is consistent with the increase in expression of class II molecules on crypt and villus epithelial cells in enteropathy a phenomenon which has been attributed to the effects of IFNγ (Barclay and Mason, 1982). This increased expression of MHC molecules may reflect an alteration in the rate of differentiation of the epithelial cells, as these molecules are only expressed on mature epithelial cells in normal animals (Steiniger et al., 1989). IFNγ also induces the expression of MHC molecules on a number of epithelial cell lines and epithelial cells *in vitro* (Basham et al., 1984, Lowes et al., 1992b, Sollid et al., 1987) and affects the proliferation of a wide range of normal cell types including keratinocytes, islet cells and thyroid cells (Trinchieri and Perussia, 1985, Symington, 1989, Nickoloff et al., '84, Weetman and Rees, 1988). The effects of IFNγ are generally cytostatic and its effects are usually enhanced by TNFα (Campbell et al., 1988).

In vivo depletion of TNFα also prevents the pathology found in mice undergoing intestinal GvHR and administering TNFα intraperitoneally to normal mice causes crypt hyperplasia and villus atrophy (Piguet et al., 1987, Garside et al., 1993). TNFα is produced mainly by activated

monocytes/macrophages (Collart et al., 1986), but can also be produced by T lymphocytes (Cuturi et al., 1987, Kobayashi et al., 1987). It is an important mediator in inflammatory responses, in particular endotoxic shock. TNFα produces fever via induction of IL1 and increases adhesion of neutrophils, monocytes and lymphocytes to endothelial cells, thus promoting infiltrates of immune cells at sites of inflammation. TNFα has been shown to stimulate the proliferation of normal fibroblasts (Vilcek et al., 1986), but has growth inhibitory or cytotoxic effects on many other cell types, including keratinocytes and tumour cells (Symington, 1989, Wang et al., 1985). Thus TNFα may be able to modulate growth of epithelial cells in enteropathy *in vivo*. TNFα synergises with IFNγ in a number of systems and mice administered with both TNFα and IFNγ have much more severe intestinal damage compared with that seen after either cytokine alone (Garside et al., 1993). The exact roles of these two cytokines are still uncertain, but *in vivo* maximum IFNγ production is associated with the peak of early proliferation, while TNFα only appears as the destructive pathology develops (Garside et al., 1994). Therefore, it is possible that IFNγ is responsible for crypt hyperplasia, while TNFα is responsible for villus atrophy.

However, the ways in which these mediators act are unknown and a number of other cytokines have also been shown to be important in experimental enteropathy. IL1 production is increased in mice with GvHR and anti-IL1α antibody partially blocks the development of pathology (Mowat et al., 1993). Administering this cytokine itself also causes crypt hyperplasia, villus atrophy and increases in IEL in normal mice. IL1β production by mononuclear cells is increased in the inflamed mucosa of patients with inflammatory bowel disease and

most of this is produced by macrophages (Mahida et al., 1989, Brynskov et al., 1992). Thus, in enteropathy there are significant amounts of IL1 present which could play an important role in the development of intestinal pathology. IL1 is a major product of activated macrophages (Collart et al., 1986, Schmidt et al., 1984) and one of its immune functions is to activate T lymphocytes (Oppenheim et al., 1986). It is also important in fever, inducing prostaglandin synthesis and acute phase protein synthesis. IL1 has been shown to stimulate the growth of fibroblasts (Dukovich et al., 1986) and, more importantly, to stimulate the growth of epithelial cells (Ristow, 1987). IL1 can also be produced in a membrane associated form by immune activated fibroblasts (Le et al., 1987, Kurt-Jones et al., 1987, Huleihel et al., 1990). Thus, IL1 as a product of activated macrophages or fibroblasts is another prime candidate for the alteration of crypt epithelial cell kinetics in enteropathy.

Other factors produced by macrophages which may affect epithelial cell turnover include IFNα/β, TGFβ (see below), IL6, and NO. IFNα/β can also induce crypt hyperplasia and villus atrophy in normal mice and stimulating production of this cytokine in mice with GvHR increases the severity of the intestinal pathology (Garside et al., 1991).

IL6 is produced by activated macrophages and T lymphocytes (Andersson and Matsuda, 1989). Although initially described as a mediator responsible for the differentiation of B cells into antibody producing cells, IL6 is now known to have a wide range of other inflammatory effects, such as the induction of acute phase protein synthesis and activation of T cells. It can stimulate the growth of keratinocytes (Krueger et al., 1990) and human intestinal epithelial

cells synthesise both IL6 and its receptor (Shirota et al., 1990). IL6 has also been shown to be over expressed in psoriatic skin another disorder characterised by hyperproliferation of epithelial cells (Krueger et al., 1990).

NO has pleiotropic effects including vasodilation and neurotransmission (Moncada et al., 1991, Nathan and Hibbs, 1991). However, NO has recently been shown to be involved in intestinal GvHR, as blocking NO production with L-NMMA prevents the development of pathology in mice with GvHR (Garside et al., 1993). As NO can be highly cytotoxic to many cell types, it is possible that this mediator could play a role in enteropathy via cytotoxic effects on epithelial cells.

Other products of T cells which may be involved in enteropathy are IL2 and IL3 and IL4. IL2, whose function appears to be activation of T cells has been shown to be present in increased levels in patients with Crohn's disease (Brynskov et al., 1992). An important function of IL3 is the stimulation of mucosal mast cells. Mucosal mast cells increase in number during experimental enteropathy (Mowat and Ferguson, 1982) and this is associated with increased levels of IL3 (Crapper and Schrader, 1986) . Although mast cells themselves do not appear to be important in enteropathy, (Newlands et al., 1990) it is possible that IL3 may have effects on other cells such as the epithelial cells. IL4 is important for B lymphocyte differentiation, but also plays a role in intestinal GvHR. *In vivo* depletion of IL4 with antibody or IL4 receptor abolishes the proliferative stage of intestinal GvHR but has no effect on the later destructive features of the disease (Mowat, unpublished).

Thus, there is evidence that a number of cytokines are involved in enteropathy and it may be that the primary effect of these cytokines is a direct action on dividing crypt epithelial cells. However, this has not been proven. Also, the full range of cytokines involved has not been identified and their mechanisms of action are not known. In addition, as will be discussed below, the regulation of epithelial cell turnover and differentiation is a very complex phenomenon. For these reasons, it is extremely difficult to dissect the mode of action or targets of cytokines in enteropathy. This is impossible to study under clinical conditions or in experimental models using intact gut *in vivo* or *in vitro*. Therefore, the aim of this thesis was to develop a simple *in vitro* model which would allow direct effects of cytokines on epithelial cells to be tested.

Control and Regulation of Growth of Intestinal and other Epithelial Cell Types

The small intestine surface is covered by a single layer of columnar epithelial cells, comprising absorptive cells and small numbers of goblet, endocrine and Paneth cells. In the crypt, there is a population of common stem cells which undergoes continuous cell division and provides constant renewal of the epithelium. Although the number of stem cells has not been determined conclusively, it has been estimated that there are between 4-40 per crypt (Potten and Loeffler, 1990). Above the stem cells is a proliferative region of epithelial cells, above which lies an upper, non-proliferating zone, where cells leave the proliferative cycle and migrate onto the villi. The basal stem cells give rise to all the different epithelial cell types (Cheng and Leblond, 1974) and are in a state of constant division; cell division occurs

throughout the rest of the proliferating zone of the crypt, but these cells also have the capacity to differentiate. At the top of the crypts, non-dividing cells migrate up onto the villus, differentiating progressively to become mature enterocytes. On reaching the top of the villus, they are extruded into the gut lumen. This process takes approximately 5-6 days in humans and 2-3 days in rodents. Under normal conditions, a steady state is maintained, with cell production in the crypts balancing cell loss into the lumen.

Possible Mechanisms for Crypt Hyperplasia

In order to determine the mechanism by which crypt hyperplasia occurs in intestinal GvHR and related enteropathies, it is important to understand the dynamics of how an increase in crypt cell production rate may occur. There are two main possibilities; the first is that crypt cell proliferation occurs as a secondary response to villus cell loss. The number of villus cells plays an important role in control of epithelial cell turnover, and an alteration in the steady state numbers of villus cells can affect proliferation in the crypt. This is evidenced by the compensatory increase in crypt cell proliferation which occurs after loss of mature villus cells caused by ischaemia or irradiation (Galjaard et al., 1972, Rijke et al., 1976). This is due to both shortening of the cell cycle time and an expansion of the normal proliferative cell compartment, resulting in a decrease of the maturation zone of the crypt. One early explanation for this influence of villus cell numbers is that mature villus epithelial cells produced factors (chalones), which normally have negative feedback effects on crypt epithelial cells and thus removal of these results in an increase in crypt cell proliferation. However, although a fraction obtained from

rabbit small intestine was shown to have anti-proliferative activity on the mouse small intestine *in vivo*, the identi ty and nature of such mediators has never been clarified (Sassier and Bergeron, 1980).

The second possibility for the basis of crypt hyperplasia is that direct stimulation of crypt cell turnover occurs. This could either be due to an increase in the proportion of proliferating cells in the crypt, an increase in the size of the proliferating zone, or a decrease in the cell cycle time. One predicted consequence of these mechanisms would be that immature cells may appear on the villus and this has been confirmed in intestinal GvHR, where lactase and aminopeptidase activities do not develop until much higher in the villus than normal (Lund et al., 1986). Although, there is also an increase in the rate of differentiation of some characteristics of enterocytes under these conditions, this cannot compensate for the more rapid migration of immature cells (Lund et al., 1986). It has been suggested that both local and systemic factors may have direct effects on crypt cell proliferative activity. That systemic humoral mediators may be involved is suggested by the fact that resection of parts of the small intestine results in a compensatory increase in epithelial cell proliferation, longer crypts and expanded villi in the remaining segments (Obertop et al., Hanson et al., 1977). These have not been identified, but hormones such as insulin, growth hormone and thyroxine play a role in regulation of intestinal epithelial growth. Removal of the pituitary gland in rats results in a decrease in crypt cell production rate and a decrease in villus height in the small intestine (Jacobson, 1964), while administration of growth hormone to hypophysectomised rats increases the mitotic rates of the crypt cells (Leblond and Carriere, 1955). Similarly, removal of the thyroid in

rats causes a decrease in crypt cell mitosis and this can be restored by addition of thyroxine (Leblond and Carriere, 1955). Thyroxine may act via stimulation of growth hormone production, but it is not yet known how these different hormones exert their effects on the intestinal epithelium.

Gastrointestinal Hormones and their Effects

Many specific gastrointestinal hormones including gastrin, secretin, cholecystokinin and enteroglucagon, have also been hypothesised to be involved in the control of epithelial cell turnover. Antrectomy of rats results in a decrease in gastrin and crypt cell proliferation and this can be restored by giving pentagastrin (Johnson and Chandler, 1973). Pentagastrin is also required to maintain some primary cultures of intestinal epithelial cells *in vitro* (Lichtenberger et al., 1973). These studies suggest that gastrin may act as a trophic hormone for small intestinal epithelium and although gastrin is unlikely to be present in the small intestine, it could still have a systemic humoral effect. *In vivo* studies have shown that secretin can inhibit the stimulatory effects of gastrin (Johnson and Chandler, 1973), although neither secretin nor choleocystokinin have direct tropic effects on intestinal mucosa in vitro (Fine et al., 1983). Bombesin has also been shown to increase DNA synthesis in colonic mucosa, suggesting that it may have trophic effects (Johnson and Guthrie, 1983), whereas somatostatin causes a decrease in DNA synthesis in the small intestine (Lehy et al., 1979). Enteroglucagon is present at increased levels in conditions which result in hyperplasia of the small intestine including coeliac disease (Besterman et al.,

1978), but there is no direct evidence that this hormone affects crypt cell turnover.

Another possible factor affecting epithelial cell turnover is the presence of foodstuffs in the small intestine. This can have a number of effects, including the direct stimulation of mucosal growth by growth factors in the food, local nutrition, and an exfoliatory effect which stimulates crypt cell production. Finally, a number of the gastrointestinal hormones can also act as neurotransmitters, including vasoactive intestinal peptide (VIP) which controls pancreatic and intestinal secretion, gastrointestinal relaxation and gut blood flow. As it also stimulates the Brunner's gland to produce EGF, VIP may be important in affecting epithelial cell turnover.

EGF has long been considered to be important in humoral regulation of epithelial proliferation in the small intestine. Produced by salivary glands and Brunner's glands in large amounts, EGF increases the proliferation of a wide number of epithelial cell types both *in vivo* (Goodlad et al., 1991) and *in vitro* (Rheinwald and Green, 1977). EGF causes an increase in crypt cell production rate and in the expression of digestive enzymes in parenterally fed rats and may have similar effects on normally fed adult mice and rats (Goodlad et al., 1991, Ulshen et al., 1986, and Al-Nafussi and Wright, 1982). EGF causes an increase in the crypt cell production rate of duodenal explants *in vitro* and increases proliferation of the RIE-1 small intestinal epithelial cell line *in vitro* (Challacombe and Wheeler, 1991, Blay and Brown, 1985). Small intestinal epithelial cells express EGF receptors and this decreases from the crypt to the top of the villus, consistent with a role for EGF in proliferation (Gallo-Payet and Hugon, 1984). Thus, EGF may be important in the physiological regulation of proliferation of

epithelial cells in the gut. However, its role as a local mediator of small bowel disease must be speculative as EGF is not produced locally throughout the small intestine.

One mediator which could fulfil this function is the closely related molecule, transforming growth factor α, which binds to the same receptor as EGF and was originally thought to be associated only with transformed cells or in early foetal development (Coffey et al., 1987 and Twardzick, 1985). However, more recently TGFα has been shown to be expressed by several normal cell types including keratinocytes and enterocytes (Coffey et al., 1987, Ciacci and Podolsky, 1991). Epithelial cells of the small intestine express mRNA for TGFα and it has been reported that there is a gradient of expression of TGFα in the small intestine of rats with higher amounts in the differentiated villus cells and lower amounts in the dividing crypt epithelial cells (Koyama and Podolsky, 1989). This suggests that TGFα is important in control of epithelial cell proliferation. Evidence that TGFα promotes the growth of the murine keratinocyte cell line BALB/MK also supports this hypothesis (Coffey et al., 1988). In addition, keratinocytes from normal skin express TGFα and patients with psoriasis have more intensely stained keratinocytes than normal patients (Gottlieb et al., 1988), suggesting that TGFα may be involved in the keratinocyte hyperplasia seen in this disease. It has also been suggested that TGFα may be an autocrine regulator of epithelial growth, as TGFα induces TGFα gene expression in normal keratinocytes and intestinal epithelial cell lines (Coffey et al., 1987, Ciacci and Podolsky, 1991). Thus, TGFα appears to be a likely mediator of proliferation in keratinocytes and intestinal epithelial cells and it is possible that factors produced

during a cell mediated immune response could influence crypt cell behaviour by altering the production of TGFα.

TGFβ is important in regulating the growth and differentiation of many cell types and is expressed widely in the intestine (Sporn et al., 1987). This pleiotropic cytokine inhibits the growth of many epithelial cell types including keratinocytes and intestinal epithelial cells, but it has a stimulatory effect on the growth of mesenchymal derived cells (Matsumoto et al., 1990, Coffey et al '88, Kurokowa et al., 1987, Massague '87). TGFβ has also been implicated in the autocrine inhibition of epithelial cell growth, but there is conflicting evidence as to its exact role, as a gradient of TGFβ mRNA expression has been reported both from the crypt to the villus and in the opposite direction (Barnard et al., 1989, Koyama and Podolsky '89). This conflict remains to be resolved, although these results suggest that TGFβ could regulate proliferation and promote differentiation of the epithelial cells. That there is autocrine control of TGFβ production by enterocytes is suggested by experiments in which TGFβ induced the expression of its own mRNA by rat intestinal epithelial cells (Ciacci and Podolsky, 1991). This was delayed by TGFα and thus these cytokines may regulate the balance between the proliferation and differentiation of epithelial cells via a feedback mechanism. An additional possible role of TGFβ is in promoting differentiation, as it has been reported that TGFβ induced the expression of sucrase in an intestinal epithelial cell line (Kurokowa et al., 1987).

The Interaction of Intestinal Epithelial Cells with the Mesenchymal Layer

The epithelial monolayer sits on a basement membrane which separates the enterocytes from the lamina propria. The lamina propria provides a supporting structure for the villus/crypt units and contains a mesenchyme of fibroblasts, smooth muscle cells and nerve fibres, as well as blood vessels and lymphatic vessels. In addition, the lamina propria contains many immune cells, including lymphocytes macrophages and NK cells. The fibroblast layer underneath the basement membrane plays a particularly important role in the regulation of growth and differentiation of enterocytes and, in the adult mouse jejunum, there is a subepithelial sheath of fibroblasts which is particularly prominent in the crypts and at the base of the villi (Marsh and Trier, 1974). During development of the small intestine in foetal rats, there is direct contact between the epithelial cells and mesenchymal cells via epithelial cytoplasmic processes (Mathan et al., 1972). These early studies suggested that a close association with fibroblasts may regulate the division and development of epithelial cells and were supported by the finding that the subepithelial fibroblasts in rabbit jejunum may migrate up the crypt and villus in parallel with the epithelial cells (Parker et al., 1974). Although this work has never been confirmed and later work suggests that the fibroblasts do not migrate, it is now clear that this interaction is of critical importance.

Additional support for the role of mesenchyme in enterocyte differentiation has come from *in vivo* developmental studies, where an intestinal epithelial cell line or embryonic endodermal cells were able to differentiate into mature absorptive cells when combined with mesenchyme (Kedinger et al., 1986). It has also been shown that mesenchyme derived from non-intestinal tissues allowed endodermal

differentiation into absorptive cells, but intestinal mesenchyme allowed differentiation into all epithelial cell types, resulting in a gut like structure (Haffen et al., 1983). This is a two way process and epithelial components also exert a morphogenetic effect on the mesenchymal cells, allowing them to organise into connective tissue and layers of muscle (Haffen et al., 1986).

Attempts to grow intestinal epithelial cells *in vitro* have reinforced the idea that fibroblasts are important for the growth and differentiation of enterocytes. Many studies have been carried out using sheets of endoderm isolated from the small intestine of foetal rats before enterocytes have appeared (Kedinger et al., 1987, Hahn et al., 1990, Haffen et al., 1981). If endoderm is cultured alone on plastic, the cells attach and spread, but do not proliferate or differentiate (Kedinger et al., 1987). However, if the endoderm is cultured on a layer of mesenchymal cells, either from the small intestine or the skin, then the epithelial cells attach and form dividing colonies which increase in size until a monolayer is formed. The resulting cells display the ultrastructural features of normal absorptive epithelial cells.

The Role of Basement Membrane in Enterocyte Differentiation

The exact role of mesenchyme is unclear, but an important aspect is the formation of basement membrane. The basement membrane is a thin sheet of extracellular matrix which separates the epithelial monolayer from the underlying mesenchyme and is made up of a complex mixture of proteoglycans and proteins such as laminin, fibronectin, collagen type IV and nidogen. The basement membrane proteins appear to be made by both mesenchymal and epithelial cells,

whereas heparan sulphate proteoglycan is produced exclusively by epithelial cells (Senior et al., 88, Simon-Assmann et al., 1988, 1989, 1990a, 1990b). The components of the basement membrane may play an important role in the regulation of enterocyte development (Simon-Assmann et al., 1990a) and compositional changes in the extracellular matrix proteins correspond with intestinal morphogenesis and differentiation (Simon-Assmann et al., 1986). The growth and survival of foetal endodermal cells is enhanced by culture on an extracellular matrix derived from Engelbreth-Holm-Swarm tumour cells and epithelial cell differentiation is induced. However, apart from isolated success using laminin, the inductive effects of intact basement membrane/mesenchyme cannot be reproduced by individual components of extra cellular matrix such as collagen type IV, fibronectin, procollagen type III and collagen type I. Thus, an intact mesenchymal layer appears to be essential for providing the optimal conditions for growth and differentiation of endoderm-derived enterocytes. It would seem likely that the mesenchyme also plays an important role in the growth of intestinal epithelial cells in mature animals.

In Vitro Models of Epithelial Cell Growth and Differentiation

The direct effects of immune cells and their products on the growth and differentiation of intestinal epithelial cells can only be studied satisfactorily in an *in vitro* model. There has been much interest in producing such models. Initially, many groups showed that explants of intact intestinal tissue from different animals could be maintained in organ cultures *in vitro* (Kagnoff et al., 1972, Black, 1978, Tsuchiya and Okada, 1982) and differentiation of foetal intestine can be

observed in some such systems (Kondo et al., 1984, Quaroni, 1985). However, as intact organ cultures contain many cell types, these systems do not provide a significant advantage over *in vivo* studies. Primary cultures of enterocytes themselves or appropriate cell lines are required for this purpose.

Primary Cultures of Intestinal Epithelial Cells

Although it is relatively easy to isolate pure enterocytes, these usually die very rapidly in culture and it has proved difficult to maintain pure cultures of intestinal epithelial cells for prolonged periods of time (Weiser, 1973, Evans et al., 1971). As it was believed initially that the isolated cells may have been damaged during extraction, later workers attempted to grow epithelial cells out of explants of intact intestine. This approach allowed several groups to grow epithelial cells from foetal or adult intestine for up to a few months and, in several cases, mature columnar cells expressing digestive enzymes were obtained (Lichtenberger et al., 1973, Lichtenberger et al., 1979, Chopra and Yeh '81, Kondo et al., 1984). However, these systems usually contained other cell types, particularly fibroblasts, thus making it difficult to assess the results obtained. These studies highlight the probable requirement for mesenchymal cells in regulating epithelial cell growth and, thus far, it has proved impossible to produce a primary culture of pure intestinal epithelial cells.

At the time I began my project, Evans and colleagues at the Patterson Institute, Manchester had recently developed a technique which appeared to allow the isolation and growth of small intestinal epithelial cells, derived from the small intestine of 6 day old rats

using enzymatic and mechanical dissociation. Under appropriate conditions, crypt/villus units can be isolated intact and attach and spread in culture. At early stages, greater than 90% of the cells stain positive for cytokeratin. However, later on in culture a greater proportion of cells which do not stain positive for cytokeratin is seen, indicating that contamination of the cultures by non-epithelial cell types occurs. Subsequent modifications of the method such as addition of heparin and alterations in the concentration of serum appeared to promote the long-term outgrowth of epithelial cells and inhibit the growth of other cell types. Thus, this technique of primary culture might provide a suitable model of small intestinal epithelial cells with dividing epithelial cells which are uncontaminated by other cell types and suitable for use in quantitiative growth assays.

Intestinal Epithelial Cell Lines

One alternative to primary culture of enterocytes is to use established lines of intestinal epithelial cells. A number of these have been established and several can differentiate *in vitro*. One of the best known is the Caco-2 human colon carcinoma cell line which differentiates spontaneously into cells with the appearance of mature small intestinal epithelial cells with a brush border containing high levels of activity of digestive enzymes (Pinto et al., 1983). The HT-29 cell line is another human colon tumour cell line which can also be induced to differentiate *in vitro*, by culture in the absence of glucose, developing a columnar appearance and a brush border expressing sucrase-isomaltase (Zweibaum et al., 1985). Although these cell lines have been used by several workers to assess the effects of cytokines on epithelial cell growth, I decided they were not appropriate for my

work for several reasons. First, they are derived from the colon, rather than the small intestine and secondly, these are tumour cell lines, whose responses to immune mediators might be unusual.

To overcome these problems several groups have attempted to establish other cell lines from normal intestinal tissue, often using explants of tissue and fibroblast feeder layers. These included the IRD 88 line and the resulting IRD 89 clone which are relatively undifferentiated epithelial cells with no brush border, although they do express a number of digestive enzymes (Negrel et al., 1983).

Quaroni et al., 1979 have also established a similar group of normal cell lines from the small intestinal epithelium. These include the IEC-6 line, which are undifferentiated cells with tight junctions, thin microvilli like structures, but no brush border (Quaroni, 1985).

Blay and Brown used similar methods to produce the RIE-1 cell line from the small intestine of a 20 day old rat (Blay and Brown, 1984). This line was isolated from intestinal fragments incubated in medium containing collagenase. After 4-6 weeks, colonies of both fibroblast and epithelial morphology had grown and an epithelioid colony with no visible contamination from other cell types was isolated, grown to confluence and subcultured serially. The resulting RIE-1 cell line has the typical morphological appearance of epithelial cells in culture, growing as tightly packed colonies of polygonal cells. Ultrastructurally, the cells are flattened, have tight junctions, thin microvillus-like structures, but no brush border and they also have a well developed endoplasmic reticulum and Golgi apparatus. They stain positively for cytokeratins and also for the epithelial specific antibody IEC1/48/1/2 (Blay and Brown, 1984). However, they do not express aminopeptidase, except at high passage numbers, indicating

that the cells are essentially undifferentiated, but may gradually acquire some features of differentiation after long periods in culture. The RIE-1 cells possess the normal rat diploid number of chromosomes up to at least passage 20, do not form colonies in soft agarose culture and do not form tumours when injected into newborn rats. Thus, they are not transformed or tumorigenic. They have high and low affinity receptors for EGF and EGF stimulates the growth of confluent cultures of RIE-1 cells, as well as acting as a chemotactic factor for their migration (Blay and Brown, 1985). For these reasons, it appears that RIE-1 cells represent undifferentiated, dividing crypt cells. As I wished to investigate the effects of immune cells and their mediators on this population, this was the principal *in vitro* model of intestinal epithelial cells I used.

Aims of this Thesis

The aim of this thesis is to test the hypothesis that products of activated immune cells directly cause the crypt hyperplasia seen in enteropathies such as coeliac disease. In addition, I wished to investigate whether these products can damage epithelial cells or affect their differentiation and thus play a role in villus atrophy. To test this hypothesis I developed *in vitro* models of small intestinal epithelial cells and examined the effects of immune cells and their products on the growth and differentiation of these cells.

CHAPTER 2

MATERIALS AND METHODS

Cell Lines and their Maintenance

The predominant cell line used in this work was the rat intestinal epithelial cell line, RIE-1, which was obtained at passage 6 from Dr. K. Brown (Cambridge). These cells were cultured in DMEM medium (Gibco, BRL, Paisley) supplemented with 5% NCS (Gibco), and the antibiotics, penicillin (Gibco) at a concentration of 100 IU/ml and streptomycin (Gibco) at 100 μg/ml. The cells were passaged weekly as described below and used until passage 20.

The L929 mouse fibroblast cell line was grown in RPMI 1640 medium (Gibco) supplemented with 5%NCS and 5% FCS (Biological Industries, Cumbernauld), 2mM glutamine (Gibco) and antibiotics. The cells were passaged twice weekly as described below.

The YAC-1 cell line, a Moloney virus induced T cell leukaemia of A strain mice ($H\text{-}2^a$), was grown in RPMI 1640 medium supplemented with 10% FCS and antibiotics. The cells were passaged twice weekly by taking 1ml of cell suspension and adding it to 9mls of fresh culture medium in a new $25cm^2$ flask (Costar).

The human colonic tumour cell line, Caco-2, was grown in DMEM medium supplemented with 10% FCS, 1μg/ml Insulin (Gibco), 1% non essential amino acids (Gibco), glutamine and antibiotics. The cells were passaged weekly as described below.

All cell culture was carried out under sterile conditions in a Laminar flow hood and sterile glass/plasticware and instruments. Unless otherwise specified, the cells were grown in 75 cm^2 tissue culture flasks (Costar, High Wycombe) and were incubated at 37°C with 5% CO_2 in a humidified Heraeus incubator.

The adherent cell lines, RIE-1, L929 and Caco-2 were passaged in the following manner:-

Once the cells were confluent, the culture medium was removed and 10mls of a trypsin/EDTA solution (0.5g trypsin/litre and 0.2g EDTA/litre in Modified Puck's Saline A)(Gibco) was added to the flask. The flask was incubated for 5mins at room temperature and then gently agitated to remove the adherent cells from the plastic. Once all the cells had detached, the suspension was pipetted into a plastic universal and centrifuged at 400g for 5mins. The supernatant was poured off and the cell pellet resuspended in 10mls of fresh culture medium. After counting in a haemocytometer (Neubauer) the final cell pellet was made up to the required concentration in the appropriate culture medium for use. Cell viability was assessed by phase contrast microscopy and was normally greater than 90%. Excess cells were seeded out into new $75cm^2$ flasks (approximately $2x10^5$ cells per flask) with 25mls of fresh culture medium and incubated at 37°C with 5% CO_2 .

Freezing and thawing of cells:-

To freeze cells for storage, $5x10^6$ cells were taken and suspended in 1ml of RPMI 1640 supplemented with 30% FCS and 5% DMSO. The cells were stored in plastic ampoules at -70°C for 24hrs before transfer to liquid nitrogen until required.

To re-culture frozen cells, the ampoule was first removed from the liquid nitrogen, thawed quickly and the contents added to 10mls of fresh culture medium pre-warmed to 37°C. The suspension was centrifuged for 5mins at 400g, the supernatant poured off and the cell pellet resuspended in 2mls of fresh culture medium. 1ml of this suspension was then placed in each of two wells in a 24 well plate (Costar) and the plate incubated at 37°C in 5%CO_2 until the cells

became confluent. The cells were then transferred into a 75cm^2 flask (Costar).

Protocols for Measuring the Effects of Cytokines on the Growth of RIE-1 Cells

To determine the effects of cytokines on the growth of RIE-1 cells in the exponential phase of growth, RIE-1 cells were suspended at a concentration of 10^5 cells/ml in culture medium and 100µl dispensed into the wells of a 96 well flat bottomed tissue culture plate (Costar). Cytokines were then added at the required concentration in 10µl of culture medium to the wells, while control wells received 10µl medium alone. The plates were covered with plate sealer (Titertek, Flow Labs, Irvine) and incubated at 37°C in 5% CO_2 before being harvested as described below. All wells were set up in quadruplicate and the mean of the 4 wells for each cytokine concentration calculated.

To determine the effects of cytokines on the growth or protein synthesis of confluent cultures of RIE-1 cells, RIE-1 cells were prepared and plated out as described above. The cells were then incubated for 3 days at 37°C in 5% CO_2, by which point the cells were confluent. At this stage the cytokines were added in 10µl of culture medium, while control wells received 10µl medium alone and the plates cultured and harvested as above.

Cytokines and Growth Factors

Epidermal growth factor - purified from mouse submaxillary glands,obtained from Sigma and diluted for storage in DMEM medium with 2% NCS at -20°C before use.

Transforming growth factor β1 - purified bovine TGFβ, from Dr. J. Carlino, Celltrix Inc., Santa Clara, California was stored in 10mM hydrochloric acid and 45% ethanol at -70°C.

Tumour necrosis factor α - recombinant human TNFα at $3x10^7$U/ml was obtained from Dr. G. Adolf, BASF, Austria and was stored in DMEM medium with 2% NCS at -70°C.

Interferon γ - recombinant rat IFNγ at $2x10^6$U/ml was obtained from Dr. G. Adolf, BASF, Austria and was stored in DMEM with 2%NCS at -70°C.

Interferon α/β - Purified mouse IFNα/β at 10^7U/ml was obtained from Lee, Biomolecular, San Diego. Stored in PBS with 2% NCS at -70°C.

Interleukin 1β - Mouse recombinant IL1β at 3.73mg/ml was obtained from Dr. A. Shaw, Glaxo, Geneva. Stored in 0.1M Tris HCl buffer at -70°C.

Interleukin 2 - Recombinant human IL2 at $2x10^5$U/ml was obtained from Biogen. Stored in PBS with 0.1% BSA at -70°C.

Interleukin 3 - Recombinant mouse IL3 at 10^5U/ml was obtained from British Biotechnology, Abingdon, Oxon and was stored in PBS with 0.1% BSA at -70°C.

Interleukin 4 - IL-4 was obtained from the supernatant of CHO cell line transfected with the rat IL-4 gene and was a kind gift of Dr. N. Barclay, Oxford. The supernatant was stored at -70°C.

Interleukin 6 - Recombinant human IL-6 at $5x10^6$U/mg of protein was obtained from C. Lawrence, Glasgow and was stored in PBS with 0.1% BSA at -70°C.

For use in experiments, cytokines were diluted immediately before use in culture medium.

Measurement of RIE-1 Cell Proliferation by Uptake of ^{3}H-TdR

The amount of DNA synthesis was determined using a method of G. Evans, Manchester. 1μCi of ^{3}H-TdR (Amersham) in 25μl DMEM medium was added to the culture wells and after further incubation for 4 hours at 37°C in 5% CO_2, the cells were rinsed twice with DMEM medium and then fixed by addition of 100μl of 100% methanol for 10 mins. The cells were then treated with a 5% solution of trichloroacetic acid at 4°C for 10 mins to remove all unbound ^{3}H-TdR, washed three times with 95% ethanol and air dried. 100μl of 0.1M sodium hydroxide solution was then added to each well and the plates incubated at 37°C for 30 mins. After neutralisation by addition of 100μl of 0.1M hydrochloric acid to each well, the contents of each well were removed and added to 5mls of liquid scintillation fluid (Eco-scint) in plastic scintillation tubes and the amount of radioactivity measured in a β scintillation counter (Minaxi). All wells were set up in quadruplicate and the average of the counts for the four wells was taken.

Measurement of Protein Synthesis by RIE-1 cells by Uptake of ^{3}H Leucine

1μCi of ^{3}H-leucine (Amersham) in 25μl DMEM culture medium was added to each culture well and after further incubation for 4hrs at 37°C in 5%CO_2 in air, the cells were washed once with DMEM medium and then incubated for 20 mins at room temperature with 200μl trypsin/EDTA solution to remove the cells from the plastic. Gentle pipetting of the trypsin/EDTA solution removed any cells which

remained adhered to the plastic. The cells were then harvested using an automatic 1205 β plate liquid scintillation counter (Pharmacia).

Measurement of Cell Growth by Uptake of MTT

The MTT colorimetric assay measures the metabolism of the tetrazolium salt 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the colured formazan product by mitochondrial succinic dehydrogenase activity in viable cells. Although, the MTT assay measures the energy metabolism of living cells, there are usually no large differences between the proliferative profiles obtained from this assay and radioisotope assays. The method of Mosmann '83 was followed.

10μl of a 10mg/ml solution of MTT (Sigma), in PBS was added to each well and after further incubation for 3 hours at 37°C in 5%CO_2, the medium was carefully removed from the wells, taking care not to disturb the blue crystals which had formed. 200μl of acid isopropanol (40ml 1M HCl in 1000ml 2-isopropanol) was added to each well and the plates incubated for 1hr at 37°C or until the crystals had dissolved completely. The absorbance of the wells at 510nm was then read using a Multiskan plate reader (Titertek). The wells were set up in quadruplicate and the average of the four calculated.

Measurement of Cell Growth by Uptake of Crystal Violet

The method of Brasaemle and Attie '88 was followed. The culture medium was removed from each well and the remaining cells washed with 1ml of Hank's Basic Salt Solution (HBSS)(Gibco) and then incubated at 4°C with 1ml of methanol for 15mins. The methanol was removed and the plates air dried. 400μl of 0.1% crystal violet (BDH,

Poole, Dorset) solution was added to each well and the plates incubated at room temperature for 5mins. The excess stain was washed off thoroughly with at least 3 washes of distilled water, the plates were dried in air and 400μl of 2% sodium deoxycholate solution was added to each well to solubilise the crystal violet. The plates were incubated in the dark at 37°C for 10-15mins until the stain was completely solubilised. A 200μl aliquot from each well was then transferred to the wells of a 96 well plate (Falcon, Glasgow) and the absorbance of the samples at 540nm was obtained using a Multiskan plate reader (Titertek).

Preparation of Activated Rat Mesenteric Lymph Node Cells

The mesenteric lymph nodes were removed from a female Wistar rat (2-3mths of age) under sterile conditions and placed in a plastic petri dish containing RPMI 1640 medium. The lymph nodes were disrupted by pushing through a plastic tea-sieve using the plunger from a 5ml syringe (Becton Dickinson) into the medium. The resulting cell suspension was then centrifuged at 400g for 5 mins, the supernatant poured off and the remaining cell pellet resuspended by gentle pipetting in medium. The cells were then counted, viability being assessed by phase contrast microscopy and viable cells resuspended at $5x10^6$/ml in RPMI 1640 (Flow) culture medium supplemented with 10% FCS, 1μl/ml $5x10^{-5}$M mercaptoethanol, 5% NCTC medium (Gibco), 100IU/ml penicillin and 100μg/ml streptomycin, and 10μg/ml concanavalin A (Sigma) and cultured in 25cm^2 tissue culture flasks (Costar). Control flasks were set up as above but omitting the concavalin A. The flasks were incubated at 37°C in 5%CO_2 for 48hrs. To obtain supernatants, the contents of each

flask were centrifuged at 400g for 5min, the supernatant collected, passed through a sterile 0.22μm filter (Sartorius, Minisart), and stored in aliquots at -70°C until required.

Preparation and Culture of Rat Peritoneal Macrophages

1 Wistar rat (2-3 months old) was injected intraperitonealy with 5mls of a 4% solution of Brewer's thioglycollate broth (Difco, E. Wolesey, Surrey). 4 days later, a peritoneal lavage was carried out on the rat under sterile conditions. The rat was sacrificed and 50mls of DMEM medium at 4°C injected into its peritoneum. The abdomen of the rat was squeezed repeatedly for 1min in order to dislodge the macrophages into the medium, which was then removed from the peritoneum using a needle and syringe and centrifuged for 5 mins at 400g at 4°C. The supernatant was discarded and the remaining cell pellet resuspended in DMEM culture medium supplemented with 5% NCS, and 100 IU/ml penicillin and 100μg/ml streptomycin. The macrophages in the suspension were counted, viability assessed using phase contrast microscopy, and viable macrophages were plated out at the required concentration in 100μl of culture medium into 96 well, flat bottomed tissue culture plates (Costar). For stimulation with IFNγ, this was added at this time in 10μl of culture medium to give a final concentration of 500U/ml and the macrophages were then cultured overnight at 37°C in 5% CO_2. The next day, the medium was removed from the wells and the adherent macrophages washed three times with DMEM to remove any debris and any remaining IFNγ. 50μl of culture medium with or without 6μg/ml LPS (Sigma) was then added to each well. The required number of RIE-1 cells in 50μl of culture medium was added to each well and all wells were brought up to a

volume of 150μl using culture medium. The plates were re-incubated at 37°C in 5% CO_2 and harvested as required.

To prepare macrophage supernatants, macrophages were isolated and cultured with or without IFNγ as described before. 75μl of culture medium with or without 2μg/ml LPS was added and the plates incubated at 37°C in 5% CO_2. After 1,2 and 3 days incubation the supernatants were removed from the wells and centrifuged at 400g for 5mins to remove any debris. The supernatants were passed through a sterile 0.22μm filter (Sartorius) and stored at -70°C until required.

Measurement of Natural Killer Cell Activity

To label target cells, $2.5x10^6$ RIE-1 or YAC-1 cells were incubated in 0.5ml of DMEM culture medium containing 50μCi of sodium^{51} chromate (Amersham) and one drop of NCS at 37°C in 5%CO_2 for 50mins. The labelled cells were then washed 5 times in fresh medium by centrifugation at 400g and, after the fifth wash, the cells were resuspended in 5 mls of medium and counted. The cells were washed once more and resuspended at a concentration of $2x10^5$ viable cells/ml.

To prepare effector cells, 1 Wistar rat (aged 2-3 months) was sacrificed and its spleen and thymus removed. The tissues were pushed through a plastic tea-sieve using the end of a syringe plunger in DMEM medium and gently pipetted up and down to obtain a single cell suspension. The cells were washed 3 times in fresh medium by centrifugation for 5 mins at 400g, resuspended in 5mls of culture medium and counted. An aliquot of $4x10^7$ viable cells was taken, centrifuged and resuspended in 2mls of culture medium.

The microcytotoxicity assays were set up in quadruplicate using a V bottomed 96 well plate (Titertek). Each well contained $2x10^4$ target cells in 100µl of RPMI 1640 culture medium and the required number of effector spleen cells in 100µl medium to give effector:target (E:T) cells of 100:1, 50:1 and 25:1. The plates were then incubated at 37°C in 5%CO_2 for 4 hrs, after which 100µl of supernatant was removed from each well taking care not to disturb the cell pellet. Each 100µl aliquot was then placed into a disposable test tube and the amount of radioactive chromium present measured using an LKB Compugamma universal gamma counter (Wallac).

The % natural cytotoxicity was calculated as follows:-

$$\% \text{ cytotoxicity} = \frac{(\text{mean c.p.m spleen} - \text{mean spontaneous release})}{(\text{maximum release} - \text{mean spontaneous release})} \times 100$$

Spontaneous release was calculated using the thymocytes at the appropriate E:T ratio, while maximum release was obtained from wells containing target cells plus 100µl 10% Triton-X 100.

Measurement of TNFα Activity by Bioassay

100µl aliquots of L929 fibroblasts resuspended in RPMI 1640 culture medium to a concentration of $2.5x10^5$ viable cells/ml were plated out in 96 well flat bottomed plates and incubated for 24 hrs at 37°C in 5%CO_2 to allow the cells to become confluent.

A set of TNFα standards was prepared by making the serial dilutions of TNFα in culture medium containing 1µg/ml Actinomycin-D

(Cosmogen) and after removing the medium from the confluent L929 cells, 100μl of each TNFα standard was added to each well. Control wells contained 100μl actinomycin-D containing medium alone or 100μl of a 10% solution of Triton-X 100 to obtain maximal cytotoxicity. Supernatants to be tested for TNFα activity were added in a volume of 10μl and the total made up to 100μl with medium containing 1μg/ml actinomycin-D. All wells were set up in triplicate, and after 24hrs culture, the number of viable adherent cells remaining were assessed by staining with crystal violet, as described above. A standard curve of cell number against TNFα concentration was obtained using the serial dilutions of recombinant TNFα and the amounts of TNFα in test supernatants was calculated from this curve.

Flow Cytometric Analysis of MHC Expression by RIE-1 Cells

RIE-1 cells were removed from tissue culture flasks as described previously, washed twice in PBS+2% NCS by centrifugation at 400g for 5mins, passed through Nitex mesh, to remove any clumps and counted.

Aliquots of $5x10^5$ cells in 100μl of PBS+2% NCS were prepared in plastic tubes and all subsequent procedures were carried out on ice, while centrifugations were performed at 4°C. To one tube, 50μl of monoclonal mouse anti-rat class I MHC antibody, OX18, (a kind gift from A. Bradley, Glasgow) was added, while class II MHC expression was detected using OX6 monoclonal antibody (a kind gift from A. Bradley, Glasgow). The cells were incubated with primary antibody for 1hr, washed twice and resuspended in 100μl DMEM medium. As a secondary antibody 50μl of 1:20 dilution of FITC conjugated F(ab)'2-rabbit anti-mouse IgG (Dako, High Wycombe, Bucks) in PBS+10%NCS

was added and the cells then incubated for 30mins. After two washes the cells were resuspended in 0.5ml PBS solution and analysed immediately using a FAC scan IV (Becton Dickinson). Control aliquots were either unstained or stained only with secondary antibody.

Preparation of Tissue Sections and Cell Cultures for Immunohistochemistry

For examination of intestinal tissue sections, 1 cm long sections were cut from the small intestine of an adult Wistar rat, snap frozen by plunging them into liquid nitrogen and placed at -70°C until required for cutting. Transverse sections of the gut, size 5μm, were cut using a cryostat (Bright Instruments Co.) and the sections carefully placed onto glass slides using a fine paint brush. These slides were wrapped in cling film and stored at -70°C until required.

For examination of cell cultures, aliquots of 10^4 RIE-1 cells in 300μl of DMEM culture medium were plated out into individual chambers of culture slides (Labtek) and incubated at 37°C in 5% CO_2 with a change of culture medium every 4 days. At the desired time points the slides of the culture chambers were snapped off, leaving the RIE-1 cells on the slides, which were washed twice with HBSS solution and then fixed at 4°C for 10 mins in acetone. The slides were air dried for 10-15 mins and then wrapped carefully in cling film and stored at -70°C.

Immunohistochemical Staining of Cultured Cells and Tissue Sections

Primary antibodies used were monoclonal mouse anti-cytokeratin at a 1:10 dilution, AE1 (ICN Immunobiologicals), monoclonal mouse IgG_1 anti-rat aminopeptidase at a 1:100 dilution (Quaroni, Ithaca, N. York), monoclonal mouse IgG_1 anti-rat lactase at a 1:100 dilution (Quaroni)

and monoclonal mouse IgG_1 anti-rat sucrase at a 1:100 dilution (Quaroni).

To detect cytokeratin staining peroxidase-conjugated goat anti-mouse IgG (Miles YEDA) was used at a 1:75 dilution, while a 1:20 dilution of FITC-conjugated F(ab)'-2-rabbit anti-mouse IgG (Dako, High Wycombe, Bucks) was used to detect enzyme staining.

The frozen sections and cultured cells to be stained were brought to room temperature and the cling film removed. The slides were fixed in acetone at 4°C for 10mins and then washed twice for 2mins in PBS. All subsequent incubations were carried out in a humidified atmosphere at room temperature unless otherwise stated. To block non-specific binding sites, 100µl aliquots of non-immunized goat serum and normal rat serum, both at a 1/10 dilution in PBS solution were incubated on the sections for 20mins. After this time, the excess serum was shaken off and the sections incubated for 2hrs with primary antibody diluted in PBS containing 0.5% bovine serum albumin (BSA)(PBS-B). The sections were then washed thoroughly three times for 2 mins in PBS and 100µl of the secondary antibody was then added at the required dilution, together with 10% normal rat serum in PBS-B and incubated for 1 hr. The sections were washed five times for 2 mins in distilled water and those sections incubated with FITC-conjugated antibody were mounted in water and viewed under a fluorescent microscope (Zeiss). Those sections incubated with peroxidase conjugated antibody were dipped twice into diamino benzidine solution [200mg DAB (Aldrich), 450ml 0.05M Tris buffered saline at pH7.4, 50ml amidazole, 250 µl hydrogen peroxide], rinsed thoroughly in distilled water and counter stained by dipping into 0.5% Haematoxylin (Orthodiagnostics), followed by Scott's tap water

substitute. The sections were dehydrated by dipping into two changes of methylated spirits, three changes of absolute alcohol and then once in xylene. The sections were mounted in Histomount (Hughes and Hughes, Romford, Essex), allowed to dry and viewed under a light microscope (Leitz).

Preparation of Type I Collagen from Rats Tails

The method of Elsdale and Bard1972 was followed. The tails were removed from 6 Wistar rats, sprayed with 70 % ethanol, the skin peeled back and the four bundles of collagen containing tendons removed. The tendons were dissolved in 400ml of 0.5 M acetic acid at 4°C for 24 hrs and debris removed by sieving, followed by centrifuging for 45 min at 3000g and 4°C. The collagen-containing supernatant was poured off and an equal volume of 20% sodium chloride solution added to precipitate the collagen. The mixture was then centrifuged for 30 min at 3000g and 4°C and the solid collagen layer removed and redissolved in 200ml of 0.5 M acetic acid. The collagen was then reprecipitated with sodium chloride solution and redissolved in 0.5M acetic acid. The acidic collagen solution was then dialysed using dialysis membrane of pore size 2-18/32" (Medicell International) against sterile distilled water at 4°C for 24 hrs and again against distilled water at pH 4 under the same conditions. The resulting solution was dispensed into sterile bottles and stored at -20°C. The concentration of collagen in the solution was calculated using a spectrophotometer, which was read at 280nm.

Isolation and Culture of Intestinal Epithelial Cells from Neonatal Rats

The method of Evans et al. 1992b was followed.

The intestines of 6 day Wistar rat pups were placed in sterile HBSS solution supplemented with 1g glucose/100mls HBSS and penicillin and streptomycin (approximately 1 animal was required per 24 well plate). Using fine scissors, the intestines were opened longitudinally and cut into 5-6 mm lengths. The segments were transferred into a 25 cm^2 tissue culture flask (Costar) with 20 mls of the HBSS-glucose solution and washed vigorously by placing on a shaking platform for 15min with at least 5 changes of HBSS-glucose solution. The intestinal segments were allowed to settle to the bottom of the flask and the HBSS-glucose solution was carefully poured off. The remaining pieces of tissue were transferred to plastic petri dishes and diced into $1mm^2$ pieces using sharp scalpel blades (Swann Morton). The diced tissue pieces were then transferred to a fresh flask containing 20 mls of HBSS solution containing 300U/ml collagenase (Sigma) and 0.1μg/ml dispase (Boehringer Mannheim) and were then shaken vigorously for a further 25mins. At the end of this period a cloudy suspension was visible and, on examination under an inverted microscope, at least a quarter of the intestinal villi had detached from the muscle layer as intact units. From this point, all procedures were carried out in a sterile cabinet with sterile plastic ware. The suspension was pipetted gently up and down for at least 3 mins through a wide mouthed plastic 10 ml pipette, transferred to a plastic universal and allowed to settle for 1 min. The top 15 mls of medium was then quickly but carefully (to prevent the sediment being disturbed) pipetted off and added to 10 mls of DMEM medium containing 5% FCS, 2% sorbital and antibiotics. The suspension was shaken for 2secs, allowed to settle for 30 secs and the pipetting and resuspending procedure repeated. The suspension was centrifuged at 300 rpm for 3 mins, the supernatant

removed carefully and the pellet resuspended in 15 mls of the DMEM-sorbital solution. This procedure was repeated at least 4 times until the supernatant became clear. The pellet was gently resuspended in DMEM supplemented with 5% FCS (Biological Industries), 20ng/ml EGF (Sigma), 0.25 IU/ml bovine insulin (Gibco), 110μg/ml sodium pyruvate, 10μg/ml porcine derived heparin (Sigma), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco).

To culture the epithelial segments *in vitro*, 200μl aliquots of 3mg/ml rat tail collagen diluted 1:10 in distilled water were placed into the wells of a 24 well plate (Costar) and allowed to dry overnight. 1ml of epithelial cell suspension was then placed in each of the collagen-coated wells. To ensure even dispersion of the cells in the wells, the cell suspension was pipetted in a gentle but continuous manner throughout the plating out procedure. The plates were then incubated at 37°C in 5% CO2.

Measurement of the Electrical Resistance of Cultured Epithelial Cells

Transwell chambers consist of small plastic inserts with a porous nitrocellulose membrane base on which cells can be cultured with separate upper and lower compartments when placed in a 24 well plate. Transwells (Costar) were precoated by adding 50μl of 1mg/ml rat type I collagen, diluted in 70% ethanol, which was then allowed to dry after removing the excess and inverting the transwell. This procedure was repeated 3 times, before the transwells were then placed into the wells of a 24 well plate (Costar).

Aliquots of $3x10^4$ RIE-1 or Caco-2 cells in 200μl of culture medium were then placed into the transwells and 800μl of culture medium placed in the bottom chamber of the well. The cells were incubated

at 37°C in 5%CO_2 and every 3 days the resistance across the cell layer was measured using an EVOM voltmeter (World Precision Instruments).

Scanning Electron Microscopy

$1.2x10^5$ RIE-1 cells in 1ml DMEM culture medium were seeded onto plastic coverslips (Thermanox) which had been placed into the wells of a 24 well plate (Costar) and grown until confluent. Taking care that the cells were covered by liquid at all times, the culture medium was removed and 1 ml of glutaraldehyde fixative, warmed to 37°C, was added to each well and the plate incubated for 1 hr at 37°C. The cells were washed three times for 5mins in phosphate/sucrose buffer and 0.5 ml of a 1% solution of osmium tetroxide in phosphate/sucrose buffer added to each well and incubated for 1 hr. The cells were then washed four times for 2mins in distilled water before addition of 1ml of a 0.5% uranyl acetate solution to each well and incubation in the dark for 30mins. The cells were washed three times for 5mins in distilled water and serially dehydrated in increasing concentrations of acetone as follows:- 10mins in 70% acetone, 10mins in 90% acetone, 3x10mins in absolute acetone and a final 10min change in dried absolute acetone. The coverslips were quickly removed from the wells, placed into a metal boat, submerged under absolute acetone and then dried in a critical point drying machine (Polaron). The samples were then mounted onto pin stubs (Philips, Agar Scientific, Stansted, Essex) using double sided sellotape, specimen side up, and the edges coated with silver paint (Agar Scientific). The coverslips were coated with gold in a Polaron SC515 SEM coating system and

then stored in a desiccator until required. The samples were viewed using a Philip's 500 scanning electron microscope.

Solutions for SEM

Glutaraldehyde fixative-10 mls 25% glutaraldehyde, 50 mls 0.2 M phosphate buffer, 38 mls distilled water, 2 mls 0.1% calcium chloride solution.

Phosphate buffer- 19 mls 0.2M potassium dihydrogen orthophosphate (KH_2PO_4), 81mls 0.2M disodium hydrogenorthophosphate, pH to 7.4.

Phosphate/sucrose buffer- 50 mls 0.2 M phosphate buffer, 48 mls distilled water, 2 mls 0.1% calcium chloride solution, 2g sucrose.

Statistical Analysis

Results are presented as means +1S.E.

Statistical significance was assessed by the Student's t test.

CHAPTER 3

THE RIE-1 CELL LINE AND ITS SUITABILITY AS A MODEL OF DIVIDING CRYPT EPITHELIAL CELLS

Introduction

The RIE-1 cells are epithelial cells derived from the small intestinal crypts of a 20 day old Wistar rat, which were isolated by Blay and Brown using the method of Quaroni (Blay and Brown, 1984). These cells are non-transformed, uncontaminated by other cell types, grow readily in culture and can be maintained for long periods of time (up to passage 20) while maintaining the same diploid chromosome number and the same morphological characteristics. As they are not polarised, do not have a brush border of microvilli and do not express digestive enzymes, these cells appear to represent immature enterocytes.

In this chapter, I assessed the suitability of the RIE-1 cell line as a model for studying the effects of cytokines and other immune products on the growth and differentiation of small intestinal crypt enterocytes *in vitro*. First, I confirmed the epithelioid nature of my RIE-1 isolate, before assessing ways of measuring its growth under different conditions *in vitro*. I then used these methods to examine if the growth of RIE-1 cells was influenced by physiological mediators known to determine epithelial cell function *in vivo*.

Results

Morphology of the RIE-1 cells

To confirm the epithelioid and undifferentiated nature of the RIE-1 cells, I initially examined its morphology using light microscopy and EM. After 2 days in culture, light microscopy showed that cells initially formed loosely associated groups (Fig. 1) which grew and coalesced to form islands. These islands eventually met to form a

complete monolayer of uniform, tightly packed, polygonal cells with the "cobblestone" appearance typical of epithelial cells (Fig. 2).

Transmission EM allowed allowed me to make a more detailed analysis of the morphological characteristics of the RIE-1 cells and to compare them with immature crypt and mature villus epithelial cells in normal rodent small intestine. As shown in figure 3, villus epithelial cells are polarised columnar cells with a well defined brush border of microvilli on the apical surface. The nucleus lies in the bottom third of the cell and the cytoplasm above is packed with organelles, a high density of endoplasmic reticulum and many vesicles. These villus epithelial cells are joined to one another by tight junctions and desmosomes. Crypt epithelial cells are more pyramidal in shape and do not have a brush border of microvilli, typical of mature cells. Although polarised to some extent, the nucleus is not situated as low down in the cell as in villus epithelial cells and the lower density of intracellular organelles is distributed more evenly throughout the cell. However, crypt cells do exhibit the tight junctions and desmosomes characteristic of epithelial cells.

RIE-1 cells were much flatter than either crypt or villus epithelial cells *in vivo* and showed no evidence of polarisation (Fig. 4). In common with crypt cells, they did not have a brush border of microvilli, but there were small microvillus-like projections on the apical surface. Although these may be rudimentary microvilli, they are smaller than villus microvilli and do not have the typical filamentous core. In addition, scanning microscopy showed clearly that these microvillus-like projections were distributed very sparsely over the cell surface unlike mature microvilli (Fig. 5). Although the RIE-1 cells had tight junctions and desmosomes typical of epithelial

cells, there was a much lower density of cytoplasmic organelles than seen in either crypt or villus cells *in vivo* .

Thus, the RIE-1 cells have some morphological characteristics of epithelial cells *in vivo*, but have no features of differentiation and most closely resemble highly undifferentiated crypt cells. However, unlike crypt epithelial cells *in vivo*, RIE-1 cells are not polarised and have fewer cytoplasmic organelles.

Expression of Cytokeratin by RIE-1 Cells

Due to the rather featureless morphology of the RIE-1 cells, I thought it important to confirm their epithelial origin by investigating whether they expressed cytokeratin. The RIE-1 cells were stained with an anti-cytokeratin antibody, AE1, which recognises most of the acidic type I cytokeratins expressed by all epithelial cells. The cytoplasm of the RIE-1 cells stained positively with this antibody and in many of the cells appeared filamentous, confirming that the RIE-1 cells are of epithelial origin. Unfortunately, these samples were destroyed in a laboratory fire, before I was able to photograph them.

Measurement of RIE-1 Cell Growth *in vitro*

As I wished to examine the effects of cytokines on the growth of RIE-1 cells, it was necessary to establish quantitative assays for measuring the growth of these cells reliably and accurately. In this experiment, I investigated the growth of RIE-1 cells using two different assays. NCS was used as a source of growth factors as this had been used by Blay and Brown 1984. The MTT assay which gives a measure of cell number, is rapid and can be used to process large numbers of samples, was my preferred choice for measuring growth

(Mosmann and Fong, 1989). I also used the uptake of ^{3}H-TdR as a well established assay for measuring proliferation and to confirm the suitability of the MTT assay.

RIE-1 cells proliferated in all serum concentrations as shown by the uptake of ^{3}H-TdR. This increased to a peak on day 3 and decreased thereafter as the cells became confluent (Fig. 6). The levels of proliferation depended on the concentration of serum in the medium, with 2.5% NCS being significantly more efficient than 1% at all times, except day 2, and 5% NCS being significantly better than 2.5% NCS on days 3 and 4 of culture.

A similar pattern was observed when cell numbers were assessed using the MTT assay (Fig. 7). Culture medium supplemented with 1% serum allowed an initial increase in cell number on days 1-3, but after day 3, there was a decrease in MTT uptake, indicating cell death. Concentrations of serum at 2.5% and 5% resulted in a steady increase in cell growth over the 4 day period, with no significant difference between the two serum concentrations. Measurement of cell number by MTT uptake correlated well with measurement of proliferation by ^{3}H-TdR uptake, as the serum-dependent increases in proliferation corresponded with increases in cell numbers and both were dose dependent.

As a result of these studies, I decided that the MTT and ^{3}H-TdR assays were suitable for assessing the growth and proliferation of RIE-1 cells and 5% NCS was chosen for use in the subsequent assays.

<u>The Effects of EGF and TGFβ on the Growth of RIE-1 Cells</u>

Having established that the RIE-1 cell line grew well in response to serum growth factors, I next examined the effects of EGF and TGFβ as representative physiological growth mediators.

As before, cells grown in serum containing medium alone showed progressive growth over 4 days, as measured by the uptake of both ^{3}H-TdR and MTT. Addition of increasing concentrations of EGF produced a significant increase in proliferation which was seen only with certain doses at the early stages of culture but was significant with all doses, except 1ng/ml, by day 4 (Fig. 8). A similar pattern was found when growth was examined by the uptake of MTT. Addition of 0.5-20ng/ml EGF to the cultures had a dose dependent ability to stimulate the growth of RIE-1 cells. This was apparent at all times of culture, although on days 1 and 2, it was only statistically significant at higher doses of EGF (Fig. 9). However, by days 3 and 4 of culture, all doses of EGF had a significant stimulatory effect on the growth of the RIE-1 cells. Despite these effects on growth, there was little change in the morphology of EGF-stimulated RIE-1 cells as assessed by light microscopy, although at the end of the culture period, the cells appeared more tightly packed in the wells than controls, presumably due to the greater number of cells in the wells.

Addition of TGFβ to the cultures had a marked cytostatic effect on the growth of the RIE-1 cells as measured by uptake of MTT. However, no dose dependent effect was apparent and maximal inhibition occurred using as little as 1ng/ml TGFβ (Fig. 10).

TGFβ also had a marked and significant inhibitory effect on the uptake of ^{3}H-TdR by the RIE-1 cells on all days and at all doses (Fig. 11). As with the MTT assay, there was no dose dependent effect and maximal inhibition was obtained using the lowest concentration.

Despite these effects on growth, there was no change in the morphology of TGFβ-stimulated RIE-1 cells as assessed by light microscopy and TGFβ did not appear to have any cytotoxic effects on the cells.

Thus, as anticipated EGF had a stimulatory effect on the growth of RIE-1 cells, whereas TGFβ had a marked cytostatic effect.

Conclusions

These experiments confirmed the epithelial origin of the RIE-1 cell line and showed that it was undifferentiated, more closely resembling crypt cells rather than mature villus enterocytes. The RIE-1 cells grew readily in conventional culture medium supplemented with 5% NCS until confluence was reached. Thus, the RIE-1 cell line appears to provide a suitable model of dividing crypt epithelial cells *in vitro*.

EGF had a dose dependent effect on the growth of the RIE-1 cells with doses as low as 0.5ng/ml having a significant effect, while RIE-1 cells were extremely sensitive to the cytostatic effects of very low concentrations of TGFβ. Thus, the RIE-1 cells responded to physiological growth factors in a manner characteristic of epithelial cells, lending further support as to their suitability as an *in vitro* model of enterocyte growth. Both the inhibitory and stimulatory effects could be measured in the presence of 5% NCS and, as this provided the best conditions for growth, this was the concentration of serum used in subsequent experiments.

The MTT and conventional ^{3}H-TdR assays correlated well with each other, with an increase in proliferation corresponding to an increase in growth. Both assays could be used to measure accurately the effects

of inhibitory and stimulatory growth mediators on RIE-1 cells, but as the MTT assay gave more consistent results, I decided to use this assay in subsequent experiments.

In the following chapters I investigated the effects of immune cells and their products on the growth of RIE-1 cells as measured by the MTT assay.

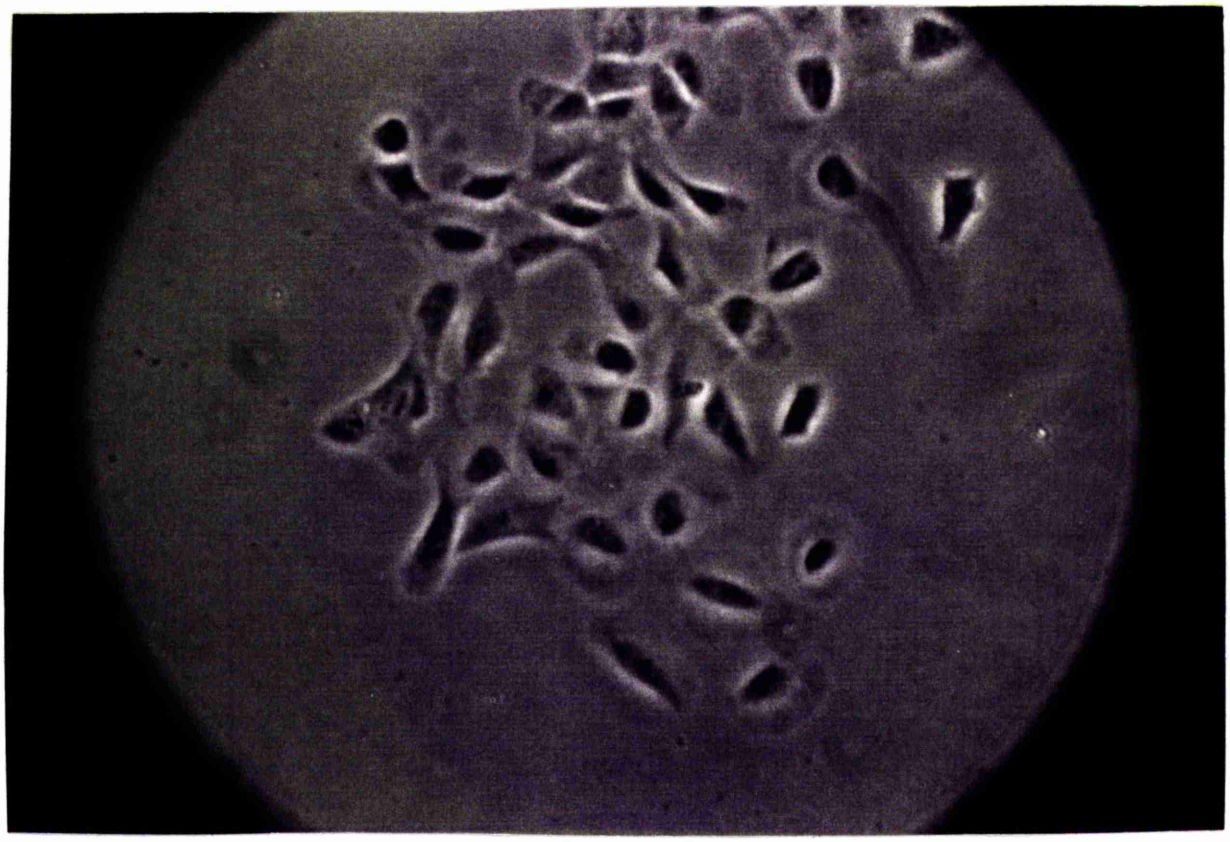

Fig 1: Group of RIE-1 cells 2 days after seeding onto plastic (X40)

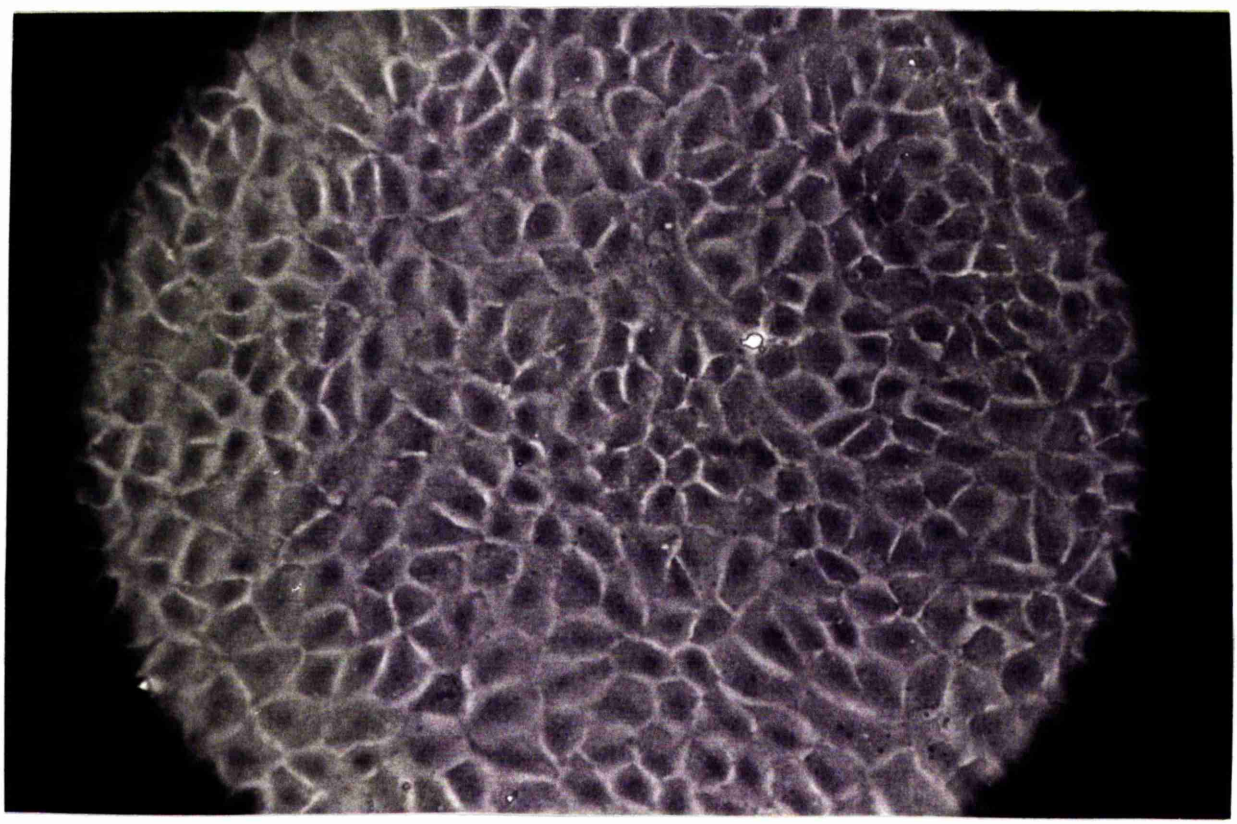

Fig 2: Confluent monolayer of RIE-1 cells, showing uniform, polygonal, tightly packed cells (X40).

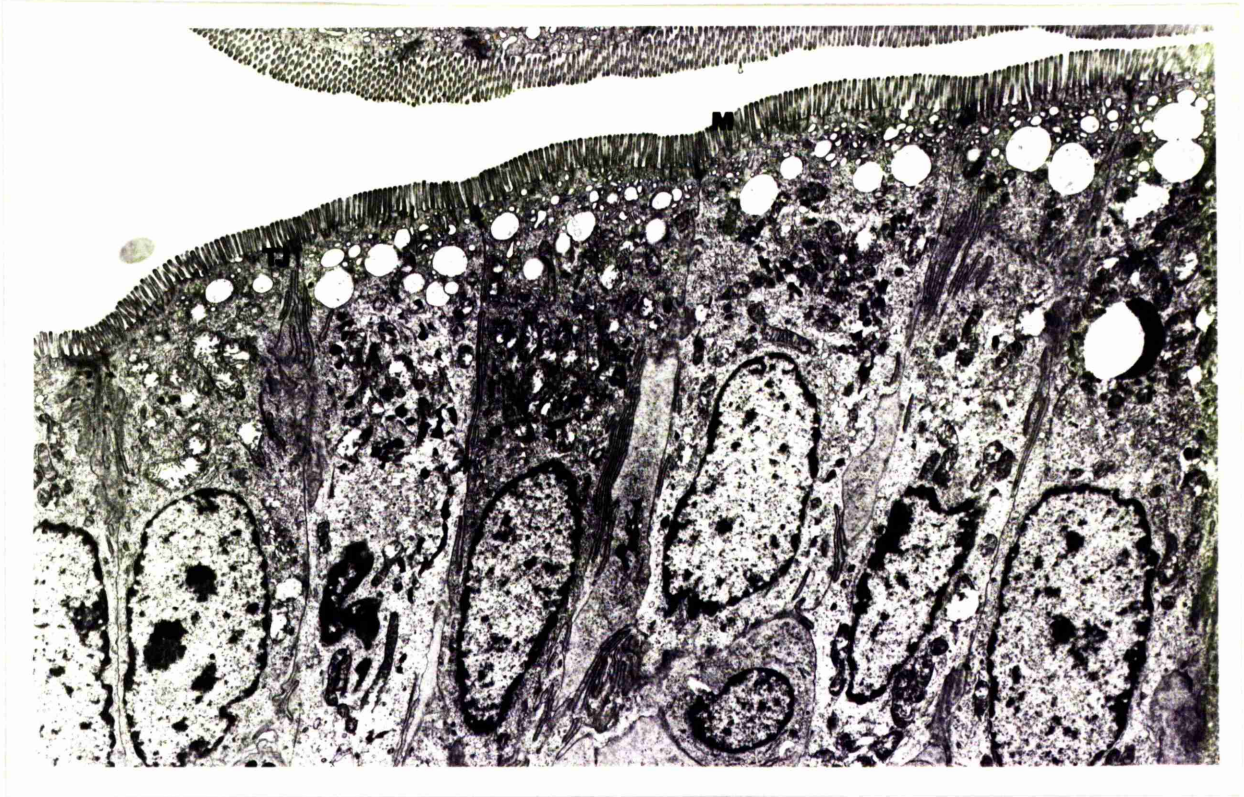

Fig 3: Transverse section through villus epithelial cells from the small intestine of a 6 day old rat, showing polarised cells with microvilli (M), tight junctions (TJ) and a high density of cytoplasmic organelles (X4400).

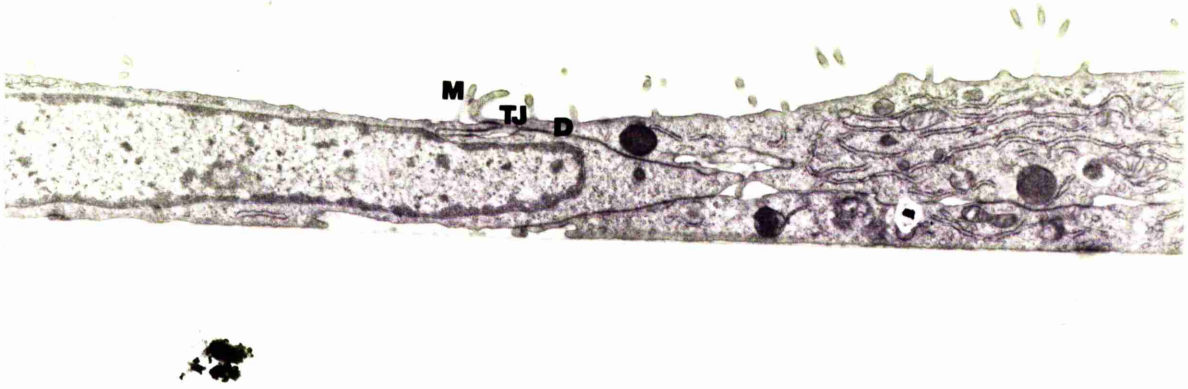

Fig 4: Transverse section through a confluent culture of RIE-1 cells, showing flattened cells with tight junctions (TJ) and desmosomes (D) and microvillus-like projections (M) (X3000).

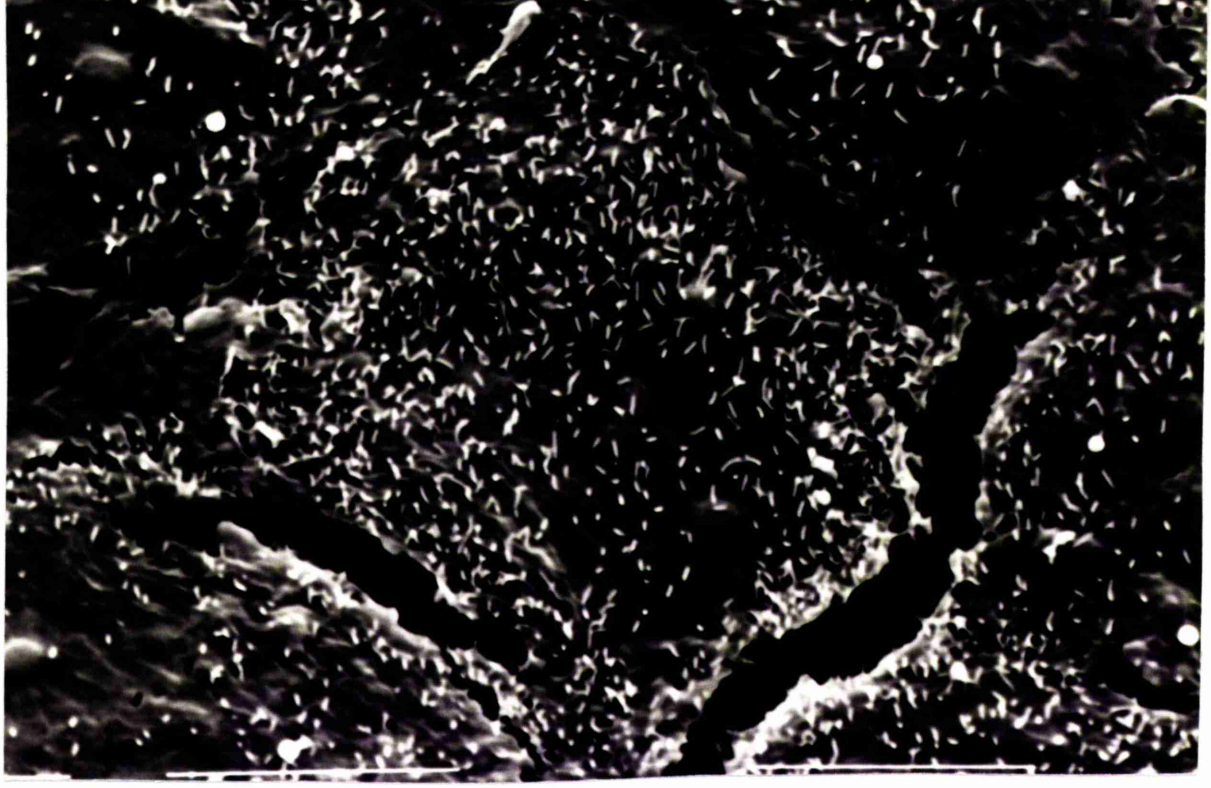

Fig 5: Scanning electron micrograph of RIE-1 cells showing the microvillus-like projections on the apical surface (X2500).

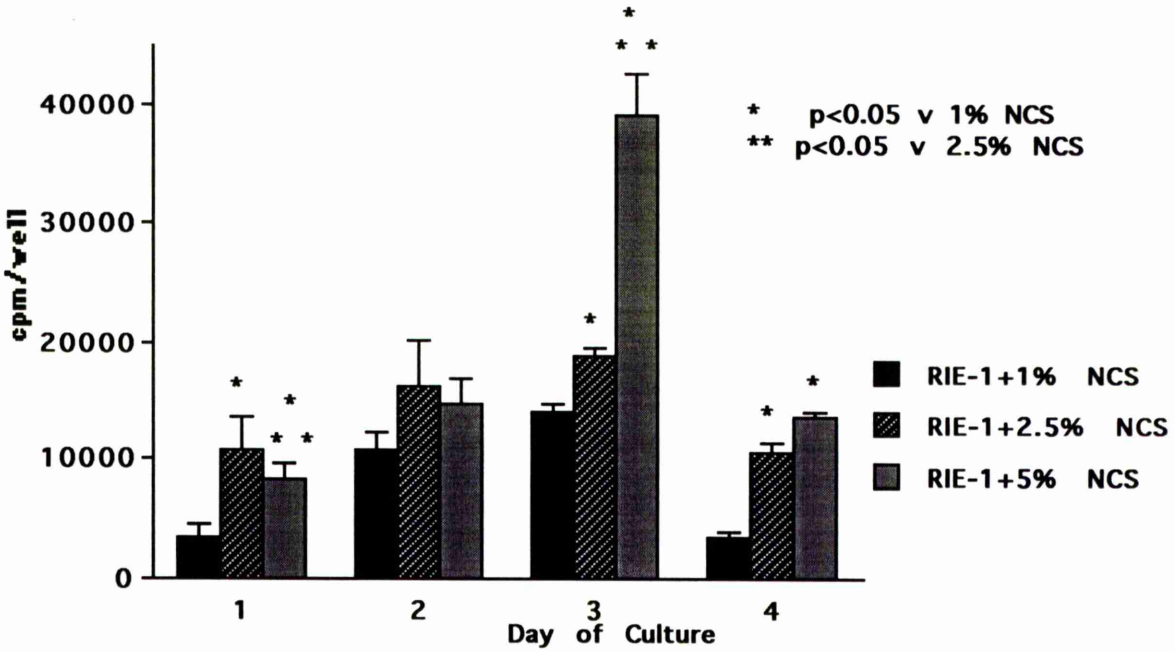

Fig 6: The effect of varying concentrations of NCS on the proliferation of RIE-1 cells as measured by uptake of tritiated thymidine.
Results shown are mean cpm/well + 1 s.e. for quadruplicate assays.

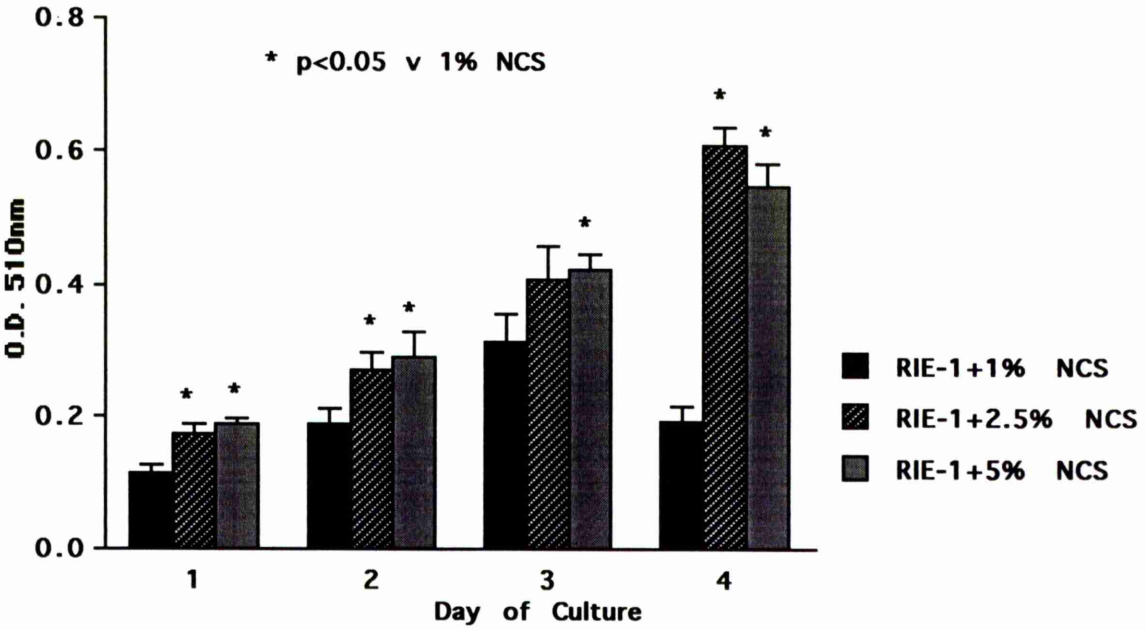

Fig 7: The effect of varying concentrations of NCS on the growth of RIE-1 cells as measured by the MTT assay.
Results shown are O.D./well + 1 s.e. for quadruplicate assays.

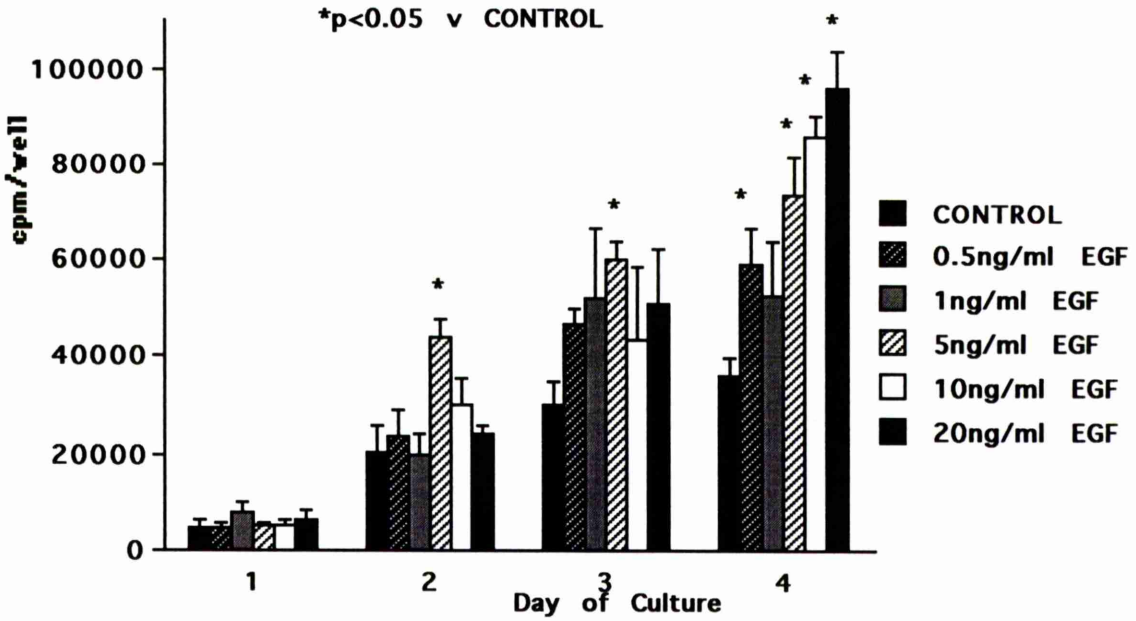

Fig 8: The effects of EGF on the growth of exponentially growing RIE-1 cells as measured by the uptake of tritiated thymidine. Results shown are mean cpm/well+ 1 s.e. for quadruplicate assays.

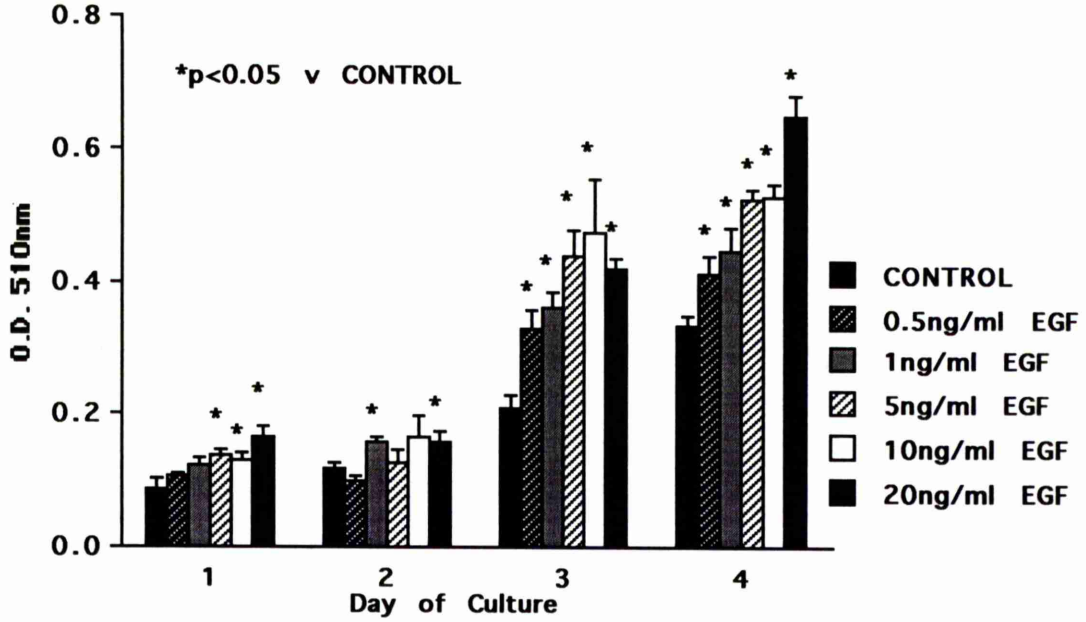

Fig 9: The effect of EGF on the growth of exponentially growing RIE-1 cells as measured by the MTT assay.
Results shown are mean O.D./well+ 1 s.e. for quadruplicate assays.

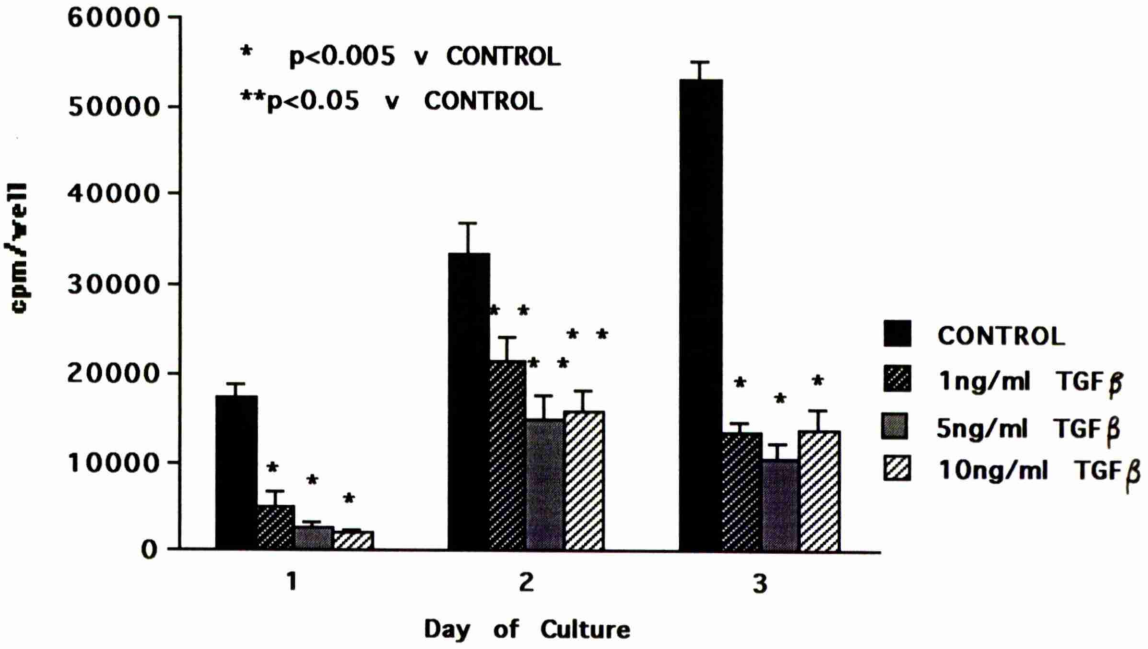

Fig 10: The effect of TGFβ on the proliferation of exponentially growing RIE-1 cells as assessed by uptake of tritiated thymidine.
Results shown are mean cpm/well+ 1 s.e. for quadruplicate assays.

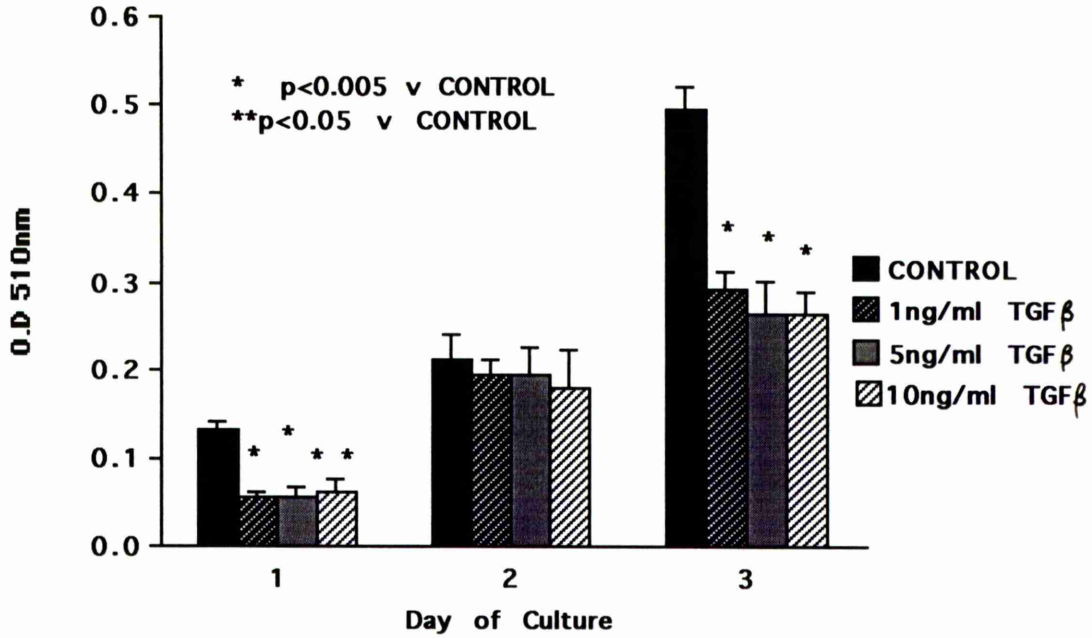

Fig 11: The effect of TGFβ on the growth of exponentially growing RIE-1 cells as measured by the MTT assay.
Results shown are mean O.D./well+1 s.e. for quadruplicate assays.

CHAPTER 4
THE EFFECTS OF IMMUNE CELLS AND THEIR PRODUCTS ON THE GROWTH OF RIE-1 CELLS

Introduction

The aim of this thesis was to investigate the hypothesis that the pathology found in enteropathies such as coeliac disease is due to a direct effect of a cell mediated immune response and its products on dividing crypt epithelial cells. In the last chapter, I established that the RIE-1 cell line might provide a suitable model of dividing enterocytes, whose growth could be modified by soluble mediators. In this chapter, I went on to investigate whether immune cells and their products could have direct effects on the growth of RIE-1 cells *in vitro*.

Initially, I looked at the effects of activated lymphocytes and their soluble products, as previous work had concentrated on the possible role of T lymphocytes in enteropathy. I then went on to examine the role of other cells involved in cell mediated immune responses, including macrophages and NK cells.

Results

The Effects of Activated MLN Lymphocytes on the Growth of RIE-1 Cells

T lymphocytes play an important role in cell mediated immune reactions and therefore I wanted to investigate whether activated T lymphocytes could have an effect on the growth of the RIE-1 cells. The MLN contains a high proportion of T lymphocytes and, in these experiments, I used ConA stimulated rat MLN lymphocytes as a source of activated T lymphocytes.

Lymphocytes isolated from rat mesenteric lymph nodes were cultured with 10^4 RIE-1 cells at ratios of 2:1 or 10:1 in the presence

or absence of 10μg/ml Con A and growth was assessed using the MTT assay.

Fig. 1 shows the effects of lymphocytes on RIE-1 cells at a ratio of 2:1. Control RIE-1 cells showed little change in number over the culture period, although the values for day 3 are significantly higher than day 1. The reason for this low growth rate is unknown. Control lymphocytes showed no increase in number, but as expected, lymphocytes activated by ConA increased significantly in number from days 1 to 2, before numbers decreased markedly on day 3 due to cell death. When lymphocytes were added to the RIE-1 cells in the absence of Con A, their MTT uptake was not significantly different from that of control RIE-1 cells, except for day 2 which was significantly higher. However, the values obtained from the mixed cells were lower on days 1 and 3, than the sum of those of the RIE-1 cells and lymphocytes cultured alone (Fig. 2). Thus, the unstimulated lymphocytes appear to have a small inhibitory effect on the growth of the RIE-1 cells.

When the lymphocytes were cultured with RIE-1 cells in the presence of Con A, the resulting MTT uptake was significantly higher than that obtained with RIE-1 cells alone on days 1 and 2, although on day 3 it was significantly lower. However, the MTT uptake in the mixed cultures was consistently less than that obtained by adding the two individual cell types together (Fig. 2). These results suggested that activated lymphocytes inhibited the growth of the RIE-1 cells.

As this was a preliminary experiment, I also investigated a 10:1 ratio of lymphocytes:RIE-1 cells. Again, lymphocytes alone did not show any significant increase in growth over the 3 days, while those activated with Con A showed a marked increase in growth which

peaked on day 2 (Fig. 3). On days 1 and 2, wells containing RIE-1 cells with control lymphocytes had MTT values which were not significantly different from those of control RIE-1 cells alone (Fig. 3), but were less than those of the two populations combined (Fig. 4). This inhibitory effect was not apparent on day 3. This inhibition was more apparent in mixed cultures containing lymphocytes and ConA, where although MTT uptake in the mixed wells was higher than that with RIE-1 cells alone (except for day 3 where there was no significant difference) (Fig. 3), this was much less than that obtained by adding the two populations together (Fig. 4). These results confirmed that activation of lymphocytes inhibited the growth of RIE-1 cells.

Effect of Soluble MLN Lymphocyte Products on the Growth of RIE-1 Cells

I then went on to determine whether activated lymphocytes released soluble products which had a similar growth inhibitory effect on the RIE-1 cells. Lymphocytes were cultured for 2 days in the presence or absence of 10μg/ml of Con A and 1,5 or 10μl of the resulting supernatant was added to RIE-1 cells in 100μl of medium. Control wells contained RIE-1 cells alone, or RIE-1 cells with supernatant from lymphocytes cultured in the absence of ConA, which had 10μg/ml of ConA added at the beginning of the culture period.

Addition of 5 or 10μl of ConA supernatant significantly inhibited the growth of the RIE-1 cells at all times of culture compared with RIE-1 cells cultured alone (except on day 1 with 5μl of ConA supernatant), an effect which was highlighted by the fact that RIE-1 cells treated with control supernatant frequently showed a significant increase in

growth compared with control cells on day 1 and 2 (10μl) or day 3 (5μl) (Fig. 5 and 6). This stimulatory effect was due to the richer medium which was used to culture the lymphocytes, as addition of 5 or 10μl of fresh medium also significantly enhanced the growth of RIE-1 cells at several times of culture (Fig. 8).

The dose dependent inhibitory effect of the ConA supernatants was confirmed by the fact that 1μl of supernatant produced much less inhibition of RIE-1 cell growth and this was only significant on days 1 and 2 (Fig. 7). Furthermore, 1μl of control supernatant had little stimulatory effect on the growth of RIE-1 cells compared with control cells. Thus, lymphocytes activated by ConA produce soluble mediators which inhibit the growth of the RIE-1 cells.

The Effect of an Anti-IFNγ Antibody on the Inhibition of RIE-1 Cell Growth by ConA Supernatant

As ConA specifically activates T lymphocytes, it seems most likely that T lymphocytes are responsible for the inhibitory effects of the MLN lymphocytes. Activated T lymphocytes produce large amounts of IFNγ and IFNγ has well documented cytostatic activity. Therefore, I thought it important to determine if IFNγ was involved in the inhibition of RIE-1 cell growth. To test this hypothesis, different concentrations of a monoclonal anti-rat IFNγ antibody was added to cultures of RIE-1 cells containing 10μl of ConA activated supernatant. As I did not know the concentration of IFNγ present in the supernatants, I used the highest concentration of antibody available, together with two lower doses. Control wells contained RIE-1 cells alone or RIE-1 cells and ConA supernatant.

As before, RIE-1 cells treated with 10μl ConA supernatant showed a significant decrease in growth compared with controls (Fig. 9). Addition of the anti-IFNγ antibody produced a dose dependent reversal of the cytostatic effect, with 200U/ml of antibody being effective on both days examined and showing a complete reversal on day 3, while 10U/ml produced significant but partial reversal on day 3. 1U/ml of antibody did not have any significant effect on the cytostasis on either day. Thus IFNγ was at least partly responsible for the growth inhibition of RIE-1 cells by ConA supernatant.

The Effect of an Anti-TNFα Antibody on the Inhibition of RIE-1 Cell Growth by ConA Supernatants

TNFα is another cytokine which is produced by activated T lymphocytes, and therefore I decided to examine its role in the growth inhibition caused by the ConA supernatant by adding different concentrations of a polyclonal mouse anti-TNFα antibody which were found to block the action of TNFα produced by activated rat macrophages (results not shown).

As before, RIE-1 cells treated with 10μl ConA supernatant showed a significant decrease in growth compared with controls (Fig. 10). None of the concentrations of anti-TNFα had any significant effect on the inhibition of RIE-1 cell growth by the ConA supernatant. These results suggest that TNFα is not involved in the growth inhibition caused by soluble MLN lymphocyte products .

The Effects of Activated Macrophages on the Growth of RIE-1 Cells

In the next series of experiments, I investigated the effects of activated macrophages and their products on the growth of RIE-1 cells.

RIE-1 cells were cultured in the presence of peritoneal macrophages which had been elicited *in vivo* with thioglycollate and restimulated *in vitro* with IFNγ, LPS, or first with IFNγ and then with LPS. IFNγ and LPS were used as they are well known activators of macrophages which together stimulate macrophages to produce cytokines such as TNFα. Control wells contained RIE-1 cells alone, RIE-1 cells cultured with LPS, and RIE-1 cells cultured with unactivated peritoneal macrophages alone. To avoid any direct effects of IFNγ, macrophages were washed after treatment with this mediator before addition to the RIE-1 cells.

RIE-1 cells cultured alone increased in number over the culture period and this was not affected by the presence of LPS (Fig. 11). The uptake of MTT by macrophages alone was low and also did not alter over the culture period.

Addition of unactivated macrophages had a significant inhibitory effect on the growth of the RIE-1 cells, but this was apparent only on day 3 (Fig. 11). Stimulation of macrophages with LPS alone did not alter this inhibitory effect, but activation of macrophages with IFNγ alone produced a dramatic and significant increase in their inhibitory effect, which was most marked on day 3 of culture. Additional stimulation with LPS had no significant effect on the cytostatic properties of IFNγ stimulated macrophages.

The inhibitory effect of unstimulated peritoneal macrophages which was enhanced by IFNγ was confirmed in several repeat experiments. Although in most cases LPS alone had no effect on either unactivated

or IFNγ-activated macrophages, in some experiments, LPS did seem to enhance the inhibitory effect of macrophages. This variation may have been due to differences in the activation state of the macrophages when isolated from the rat. There was no evidence of phagocytosis or cytotoxicity by macrophages in any of the wells as examined by light microscopy.

The Effect of Blocking Prostaglandin Synthesis on the Inhibition of RIE-1 Cell Growth by Macrophages

I then went on to investigate the basis of the growth inhibitory effects of macrophages on epithelial cells. Prostaglandins are important inflammatory products of activated macrophages, which are growth inhibitory to many cell types. To examine whether prostaglandins contributed to the effects of macrophages on RIE-1 cells, different concentrations of the prostaglandin synthase inhibitor, indomethacin, were added at the beginning of the macrophage preculture.

At the doses used indomethacin had no effect on the growth of the RIE-1 cells (Fig. 12a). As before, addition of unactivated macrophages had a small but significant inhibitory effect on the growth of the RIE-1 cells as shown in the previous experiment, and this was enhanced by stimulation with IFNγ, but not LPS (Fig. 11). Addition of indomethacin did not prevent the inhibition induced by unactivated macrophages or macrophages activated with IFNγ alone (Figs. 12b and 12c). Similarly, indomethacin had no significant effect on the growth inhibition caused by LPS stimulated macrophages (Fig. 12d). The highest dose of indomethacin also had no significant effect on the inhibitory effects of macrophages activated by LPS and IFNγ (Fig. 12e).

However, in the experiment shown, the two lowest doses caused partial but significant reversal of the inhibition. This result appeared to be anomalous as it was not obtained in repeat experiments and I concluded that prostaglandins do not contribute to the growth inhibitory effects of macrophages on RIE-1 cells.

The Effect of Blocking NO synthesis on the Inhibition of RIE-1 Cell Growth by Macrophages

I next investigated whether NO played a role in the cytostatic effect of macrophages. NO production has been implicated in several aspects of macrophage function (Nathan and Hibbs, 1991) and is required for intestinal GvHR (Garside et al., 1992). It is also inhibitory to several cell types. To assess the role of NO, the specific inhibitor of macrophage dependent NO synthase, L-NMMA, was added to the co-cultures at the beginning of the culture period. On day 2 of this experiment, macrophages activated with IFNγ or both IFNγ and LPS had a marked cytostatic effect on the growth of the RIE-1 cells, while unactivated macrophages and macrophages activated by LPS had no significant effect on the growth of the RIE-1 cells (Figs. 13a-d). However, as in previous experiments, all the macrophage populations significantly inhibited the growth of the RIE-1 cells by day 3.

Blocking NO production with L-NMMA had rather inconsistent effects on the cytostatic properties of the different populations of macrophages. L-NMMA had no effect on the growth inhibition produced by unstimulated or IFNγ activated macrophages at either time point (Fig. 13a and b), nor on the cultures harvested on day 2 using macrophages activated with LPS or with LPS and IFNγ (Fig. 13c and d). However, on day 3, there was a small but significant reversal

of the growth inhibition caused by LPS and IFNγ activated macrophages and a larger reversal of the growth inhibition produced by macrophages stimulated by LPS alone. Thus, NO may contribute to the growth inhibition of RIE-1 cells caused by LPS, or LPS and IFNγ stimulated macrophages.

The Role of Blocking TNFα in the Inhibition of RIE-1 Cell Growth by Macrophages

A major inflammatory product of activated macrophages is TNFα and it is known that this cytokine is cytotoxic or cytostatic to a range of cell types. Therefore I examined whether TNFα could account for the cytostatic effects of macrophages on RIE-1 cells.

First, I assayed the levels of TNFα in supernatants from the different populations of activated and control macrophages using a bioassay. Unstimulated macrophages and macrophages activated by IFNγ did not produce TNFα, whereas macrophages which had been activated by LPS alone or LPS and IFNγ produced measurable amounts of this cytokine (results not shown). To investigate whether this TNFα contributed to the inhibitory effects of these macrophages, I examined whether the cytostatic effect could be blocked by an anti-TNFα antibody. Co-cultures of activated macrophages and RIE-1 cells were set up in the presence of 1μl or 5μl of an anti-mouse TNFα polyclonal antibody. The growth of cells in the absence of antibody has already been shown in Fig. 11, where it was found that inhibition of RIE-1 cell growth by macrophages occurred. Both concentrations of antibody partially, but significantly reversed the cytostatic effect of LPS stimulated macrophages on day 3 (Fig. 14a). Both concentrations of the antibody also partially and significantly reversed the cytostatic

effect of macrophages activated by LPS and IFNγ. Addition of the antibody to the RIE-1 cells alone had no effect on their growth (Fig. 14b). Thus, TNFα may be at least partly responsible for the inhibititory effects of macrophages activated with LPS alone or with IFNγ and LPS.

The Effect of Macrophage Supernatants on the Growth of RIE-1 Cells

The results described above provide some evidence that macrophage-derived mediators may be responsible for the cytostatic effect of macrophages on RIE-1 cells. As many macrophage functions require cell-cell contact, I thought it important to examine the possible role of soluble mediators further by investigating the effects of soluble products of the different macrophage populations on RIE-1 cells. Peritoneal macrophages were prepared and stimulated with IFNγ and/or LPS *in vitro* as described before and supernatants harvested 1, 2 and 3 days later. 10μl of supernatant was then added to RIE-1 cells in 100μl of medium.

Day 1 macrophage supernatants:

Supernatants taken from macrophages stimulated for 1 day *in vitro* generally had no effect on the growth of the RIE-1 cells, except for those supernatants derived from macrophages which had been activated with LPS (Fig. 15a). These supernatants had a small but significant stimulatory effect on growth on day 1, but by day 3, this effect was reversed and there was now significant inhibition of growth.

Day 2 macrophage supernatants:

Macrophage supernatants collected after 2 days stimulation gave somewhat different results. Again, supernatants from macrophages

activated with LPS had a small but significant stimulatory effect on growth of the RIE-1 cells on day 1, which disappeared after this time and a significant inhibitory effect was observed on day 3 (Fig. 15b). Supernatants from unstimulated macrophages now also produced significant inhibition of growth on day 2 of culture. The supernatants from macrophages activated with IFNγ, or IFNγ and LPS also had significant inhibitory effects on the growth of the RIE-1 cells and this was apparent on day 3 for the former, and on both days 2 and 3 for the latter.

Day 3 macrophage supernatants:

As before, supernatants from macrophages activated with LPS for 3 days had a significant stimulatory effect on the growth of RIE-1 cells on day 1 of culture, but produced significant inhibition of growth by day 3 (Fig. 15c). Supernatants from macrophages stimulated with IFNγ and LPS produced the same pattern of enhanced growth on day 1, followed by inhibition on day 3. However, the inhibitory effect on day 3 was not as marked as with LPS alone. Supernatants from unstimulated macrophages caused a small inhibition of growth which was significant on day 2. Supernatants from macrophages stimulated with IFNγ for 3 days had a pronounced inhibitory effect on RIE-1 cells which was evident on days 2 and 3 of culture.

Cytotoxicity of NK Cells to RIE-1 Cells

The final population of cellular effector cells which I examined was NK cells. These cells are involved in a number of cell mediated immune responses *in vivo*, including intestinal GvHR and regulate the growth and differentiation of several cell types. Rat spleen cells were used as effectors in conventional ^{51}Chromium release

microcytotoxicity assays, using RIE-1 cells and YAC-1 cells as targets. NK cells had no cytotoxic effects on the RIE-1 cells, but had excellent cytotoxic activity against the YAC-1 cells, which were used as a positive control (Table 1).

Conclusions

These experiments have shown that immune cells and their products can have direct effects on the growth of a dividing crypt epithelial cell line. The MLN contains high numbers of T lymphocytes and ConA stimulated MLN lymphocytes inhibited the growth of RIE-1 cells. Although resting lymphocytes also had some effect, this was much less than that found using activated cells. Although these findings are consistent with the possibility that activated T lymphocytes can regulate enterocyte growth, it should be noted that my studies do not prove directly that T lymphocytes were the cells responsible for the activity of the MLN lymphocytes. The inhibitory effect seen in the mixed cultures of MLN lymphocytes and RIE-1 cells was not merely due to competition for space and nutrients, as supernatants from the ConA activated MLN lymphocytes also had a marked cytostatic effect on the growth of the RIE-1 cells. In contrast, control MLN lymphocyte supernatants had no effect. The effects of the ConA supernatant appeared to reflect true cytostasis, as the cells appeared healthy and there was no evidence of cytotoxicity. The inhibitory effect of these supernatants was at least in part caused by IFNγ, while TNFα, another T lymphocyte product with cytostatic properties, did not appear to contribute to the growth inhibition of RIE-1 cells caused by MLN supernatants.

Having demonstrated that T lymphocytes and their products had direct effects on RIE-1 cell growth, I then investigated whether activated macrophages might have similar effects. Thioglycollate elicited peritoneal macrophages inhibited the growth of RIE-1 cells and this effect was enhanced by further activating the macrophages with IFNγ. However, additional stimulation with LPS had no effect on the inhibitory action of either unstimulated or IFNγ stimulated macrophages. Macrophages which had not been restimulated *in vitro*, were also slightly inhibitory to RIE-1 cells. This could reflect the stimulatory effects of thioglycollate or that the macrophages were already partially activated *in vivo*, perhaps via the effect of gut-derived LPS.

I then proceeded to investigate the nature of the growth inhibitory effects caused by the different populations of macrophages. Again these did not appear to be due to cytotoxic or phagocytic activity, as under light microscopic examination, the cultures of RIE-1 cells remained healthy, with no evidence of cell loss.

Prostaglandins were not required for the inhibitory effect, as blocking their production with indomethacin had no effect on the growth inhibition caused by any of the macrophage populations. In addition, although NO appeared to play some role, this was only with macrophage populations which had been activated by LPS or IFNγ/LPS. TNFα was also produced only by LPS and LPS/IFNγ stimulated macrophage populations and the cytostatic effects of these cells was partially reversed by an anti-TNFα antibody. However, it is important to emphasise that LPS stimulated macrophages had no greater inhibitory effects than unstimulated macrophages and that none of these mediators had any effect on the inhibition caused by

IFNγ stimulated macrophages, whose inhibitory effects on RIE-1 cells were by far the greatest when the populations were compared. These experiments suggest that the LPS stimulated macrophages cause cytostasis by different mechanisms from those exerted by IFNγ stimulated macrophages.

To investigate the role of macrophage-derived soluble mediators further, I examined the effect of supernatants from the different macrophage populations on the growth of RIE-1 cells. These supernatants also inhibited growth and, and in general, the results correlated with those using macrophages themselves in the cultures. However, the effects of the supernatants differed, depending on the day on which they were harvested. Apart from those derived from macrophages stimulated by LPS, all supernatants harvested on day 1 of culture had no effect on growth. This finding correlated well with those using the macrophages themselves, which also had no effect after culturing for one day with RIE-1 cells. The supernatants harvested on days 2 and 3 of culture mainly had inhibitory effects, and stimulation of the macrophages with LPS and/or IFNγ enhanced the growth inhibitory effects of RIE-1 cells, which was again consistent with the macrophages themselves.

One contrasting feature was that supernatants from macrophages stimulated with LPS had a biphasic effect which was not evident using LPS stimulated macrophages themselves. Initially, these supernatants had a stimulatory effect on the growth of RIE-1 cells, but later times an inhibitory effect was seen and this was as marked as that caused by supernatants from IFNγ stimulated macrophages. The absence of this biphasic effect when using macrophages themselves may have

been due to a lower sensitivity of the assays when applied to co-cultures, in which both cell types can take up MTT.

Together, the experiments using both T cells and macrophages point to a role for soluble immune mediators in the regulation of epithelial cell growth. However, the results from mixed cell cultures and supernatants did not always correlate, while the effects of blocking antibodies or other inhibitors was rarely complete. There may have been a number of reasons for this, including the fact that I used unseparated preparations of MLN cells and that at least some of the macrophages may have already been partially activated *in vivo*. As a result, the effects of the cell populations and supernatants observed here probably reflect the involvement of a number of different cytokines and cell types. For these reasons, in the next chapter I investigated the effects of purified, individual cytokines on RIE-1 cell growth.

NK cells have been shown to be important in intestinal GvHR, but in my experiments, NK cells were not cytotoxic to the RIE-1 cells in a chromium release cytotoxicity assay. Nevertheless, this does not exclude the possibility that activated NK cells could contribute to the effects on epithelial cell growth by releasing cytokines.

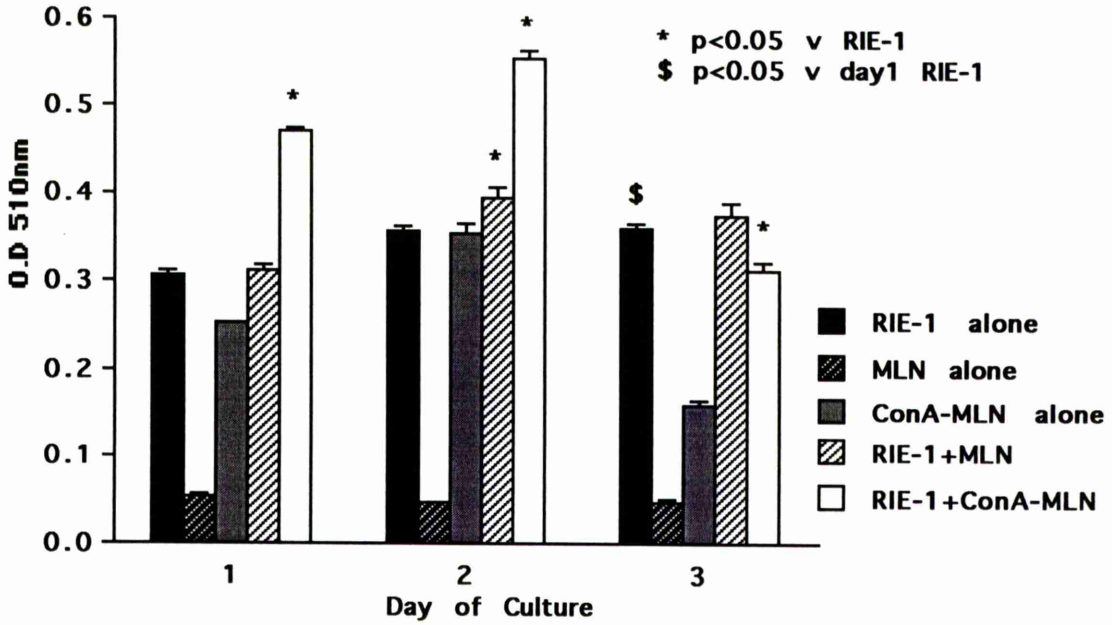

Fig 1: The effect of unactivated and Con A activated MLN cells on the growth of RIE-1 cells at a ratio of 2:1 respectively as measured by the MTT assay. Results shown are O.D./well +1s.e. for quadruplicate assays.

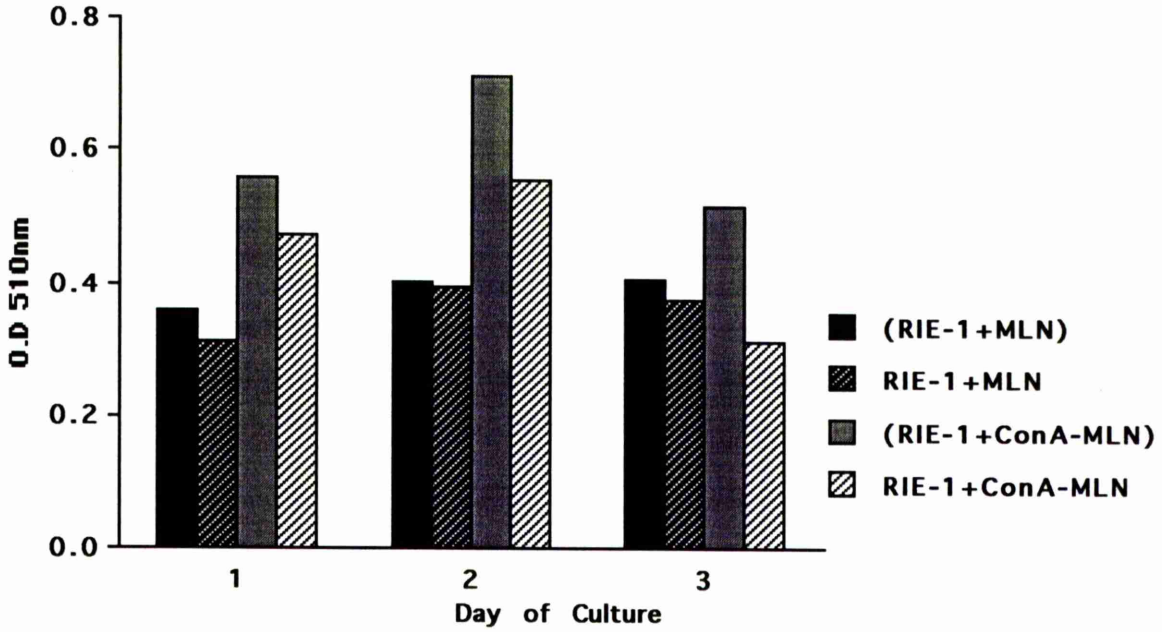

Fig 2: Comparison of the values of mixed cultures of RIE-1 and MLN cells and the sum of the values of RIE-1 and MLN cells [denoted by ( )] at a ratio of 2:1. No statistics were possible.

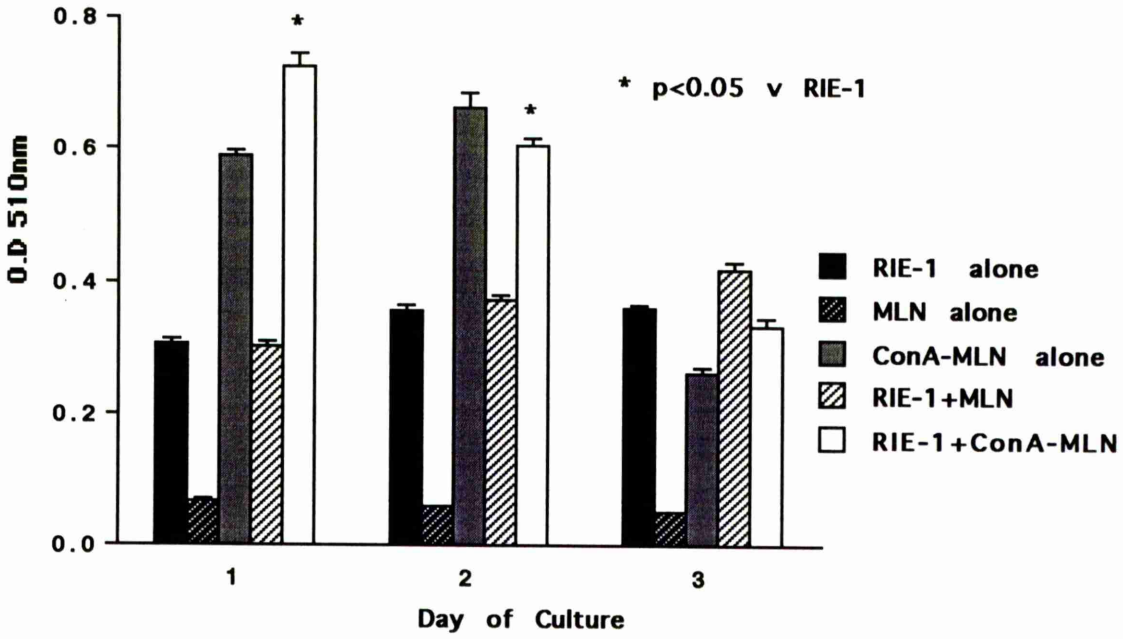

Fig 3: The effect of unactivated and Con A activated MLN cells on the growth of RIE-1 cells at a ratio of 10:1 respectively as measured by the MTT assay. Results shown are O.D./well +1s.e. for quadruplicate assays.

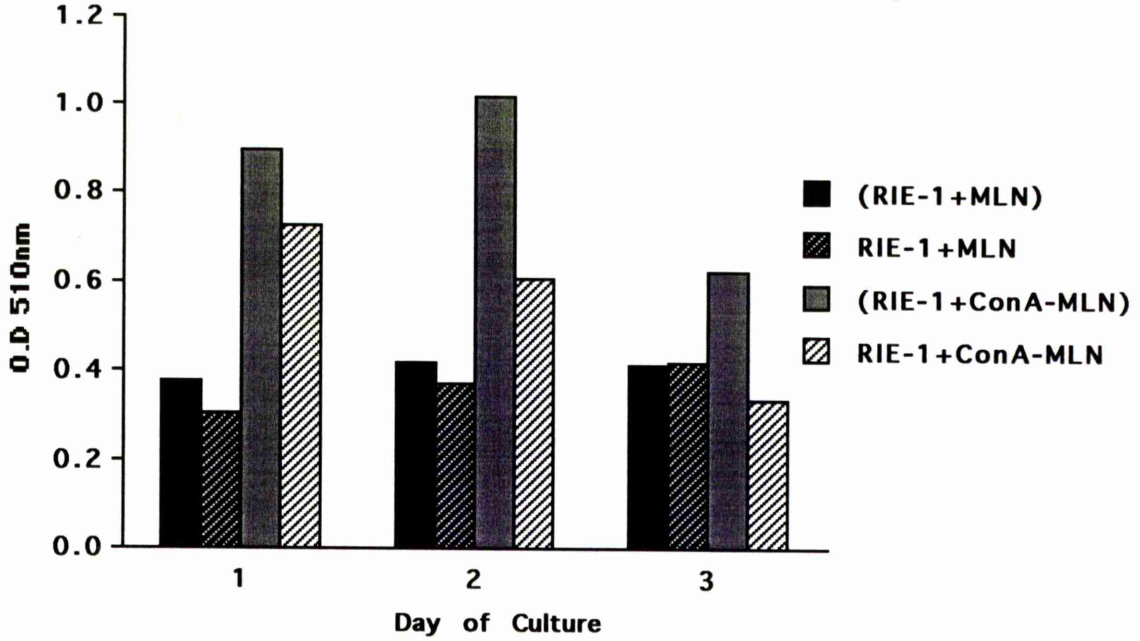

Fig 4: Comparison of the values of mixed cultures of RIE-1+MLN cells and the sum of the individual values of RIE-1 and MLN cells [denoted by ( )] at a ratio of 10:1. No statistics were possible.

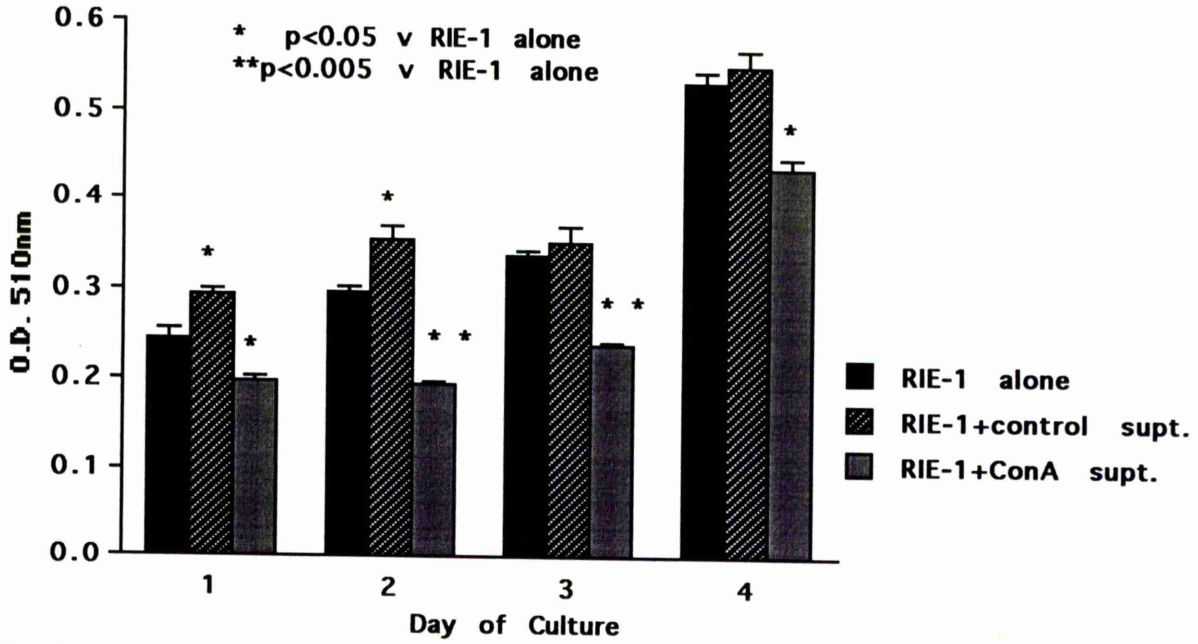

Fig 5: The effect of 10µl of supernatant from unactivated and ConA activated MLN cells on the growth of RIE-1 cells as measured by the MTT assay. Results shown are O.D./well+1s.e. for quadruplicate assays.

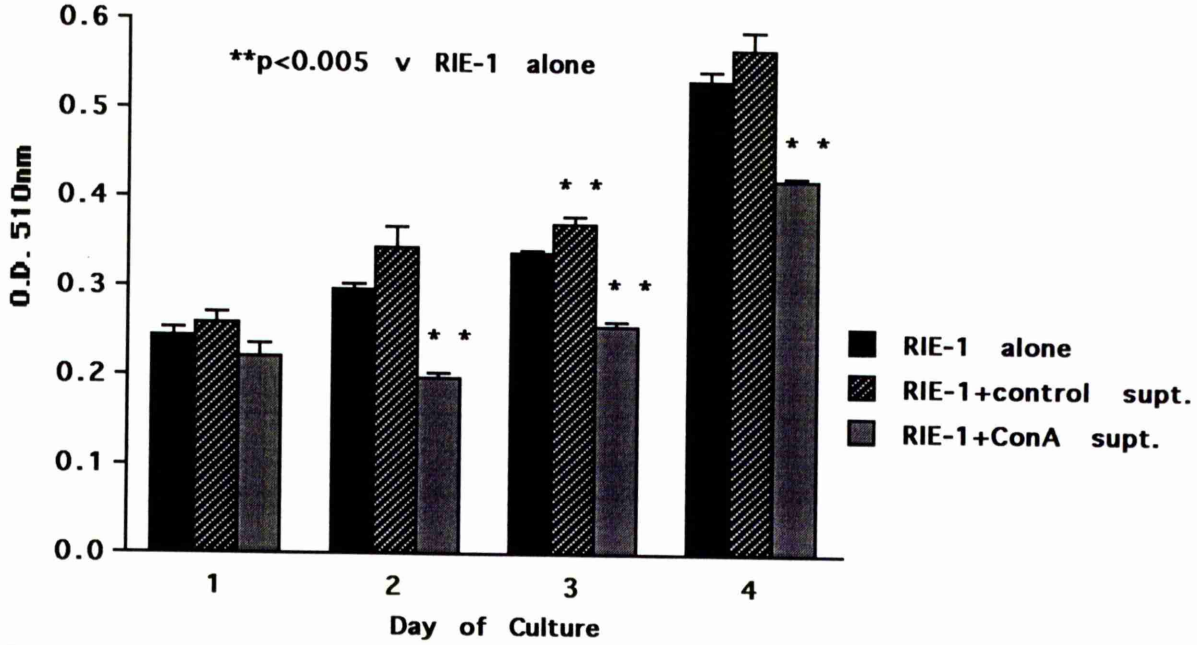

Fig 6: The effect of 5µl of supernatant from unactivated or ConA activated MLN cells on the growth of RIE-1 cells as measured by the MTT assay. Results shown are O.D./well+1s.e. for quadruplicate assays.

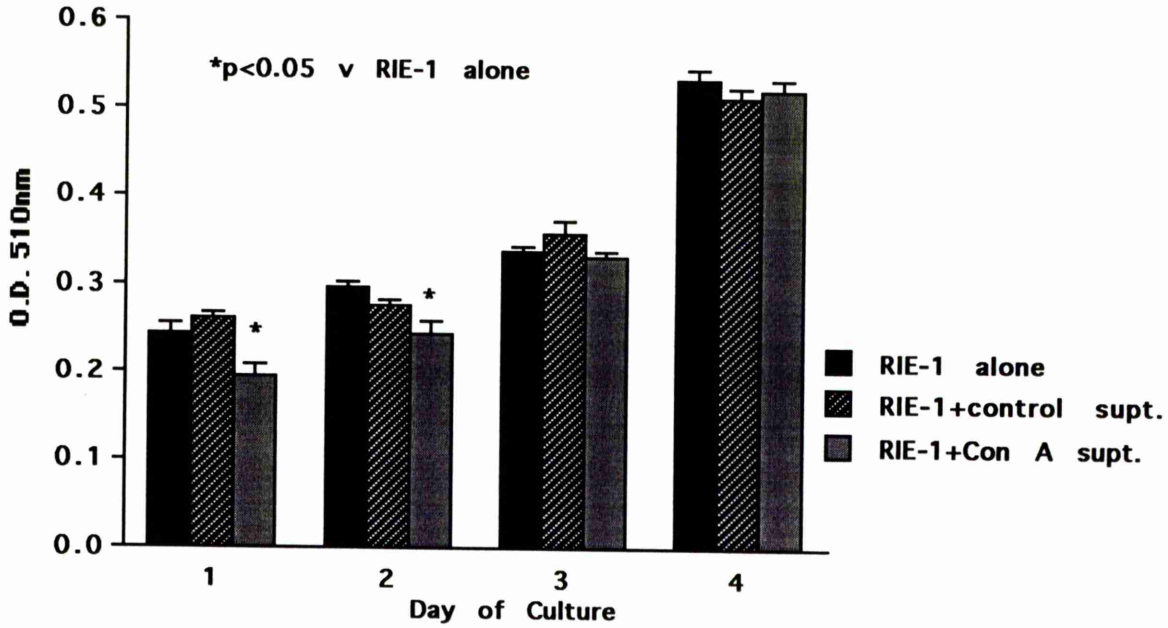

Fig 7: The effect of 1μl of supernatant from unactivated or ConA activated MLN cells on the growth of RIE-1 cells as measured by the MTT assay. Results shown are O.D./well+1s.e. for quadruplicate assays.

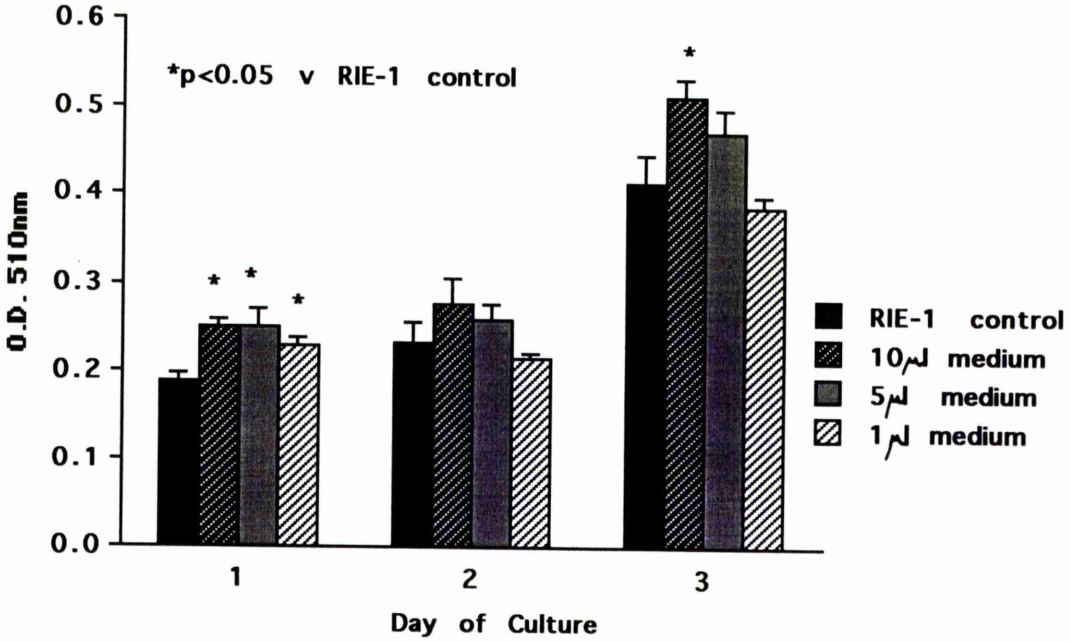

Fig 8: The effect of MLN cell culture medium on the growth of RIE-1 cells as measured by the MTT assay.
Results shown are O.D./well+1s.e. for quadruplicate assays.

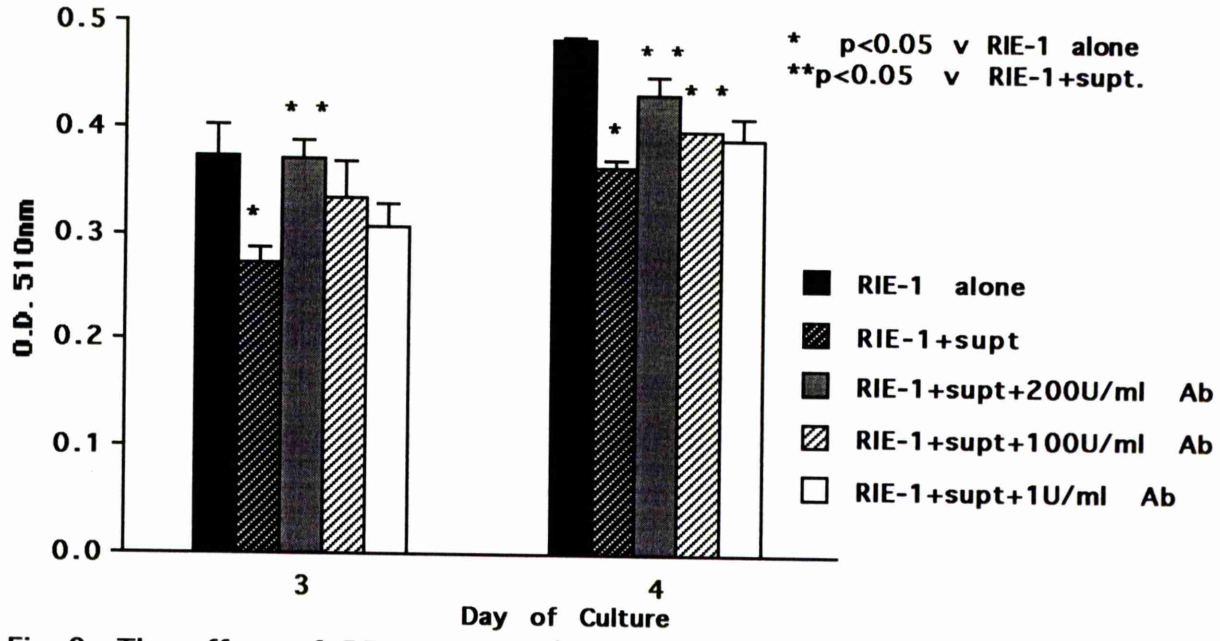

Fig 9: The effect of DB-1 anti-IFN γ antibody on the growth inhibition of RIE-1 cells caused by 10μl supernatant from ConA activated MLN cells, as measured by the MTT assay.
Results shown are O.D./well+1s.e. for quadruplicate assays.

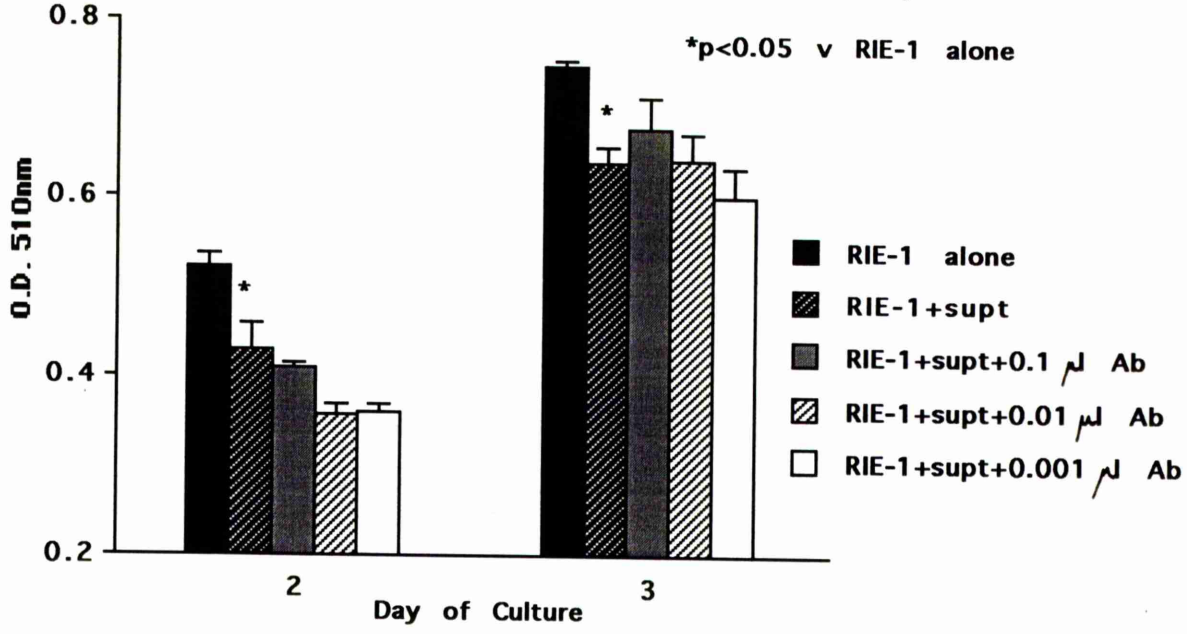

Fig 10: The effect of an anti-TNF α antibody (E. Liew) on the growth inhibition of RIE-1 cells by supernatant from ConA activated MLN cells as measured by the MTT assay. Results shown are O.D./well+1s.e. for quadruplicate assays.

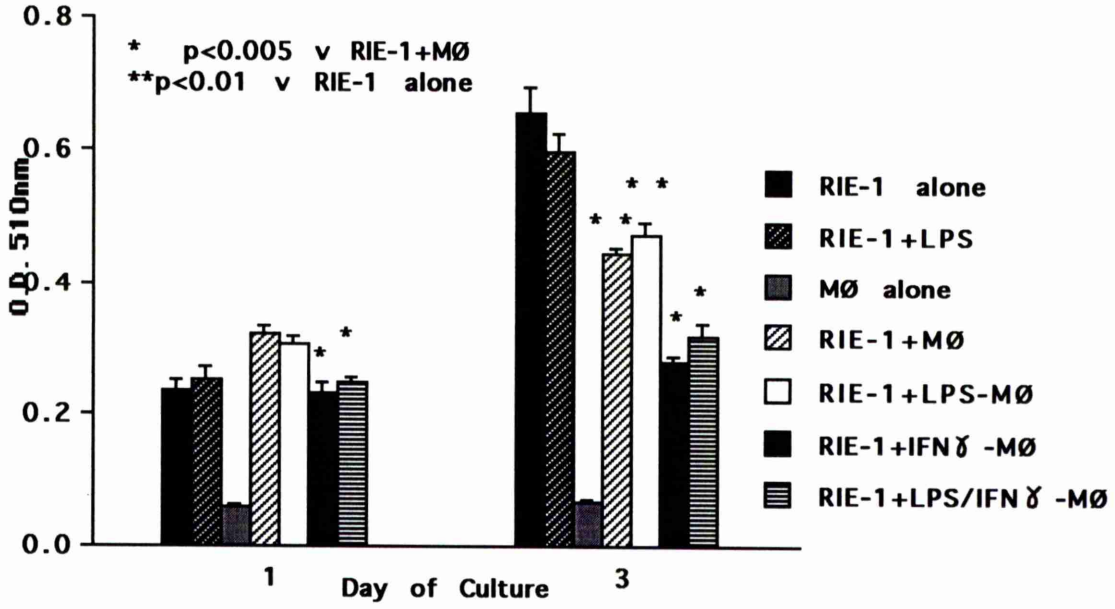

Fig 11: The effect of macrophages on the growth of RIE-1 cells as measured by the MTT assay.
Results shown are O.D./well+1s.e. for quadruplicate assays.

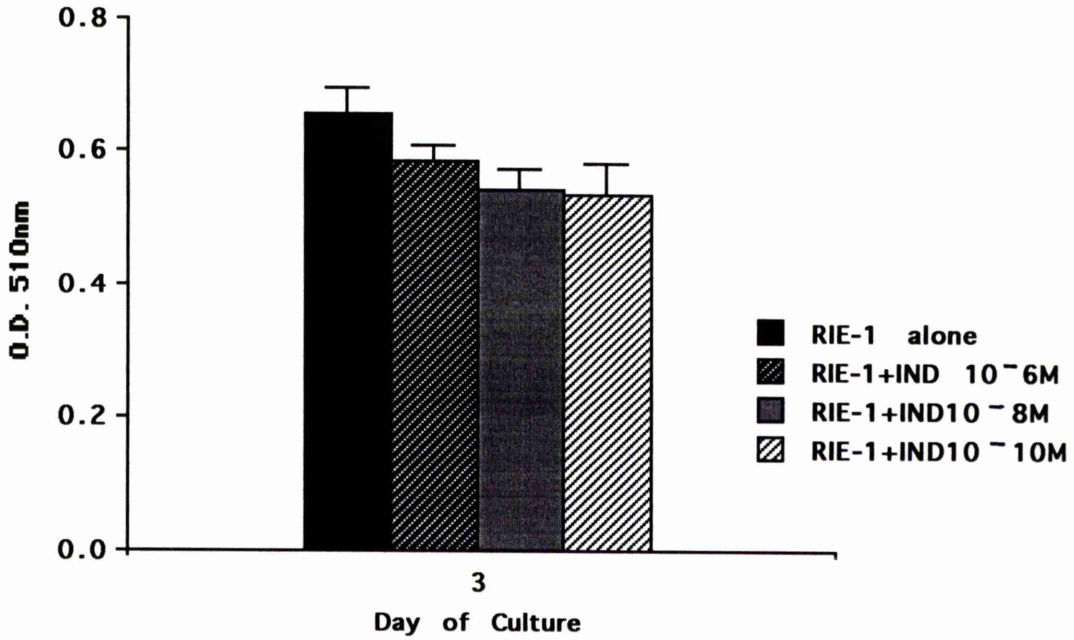

Fig 12a: The effect of indomethacin on the growth of exponentially growing RIE cells as measured by the MTT assay.
Results shown are O.D./well+1s.e. for quadruplicate wells.

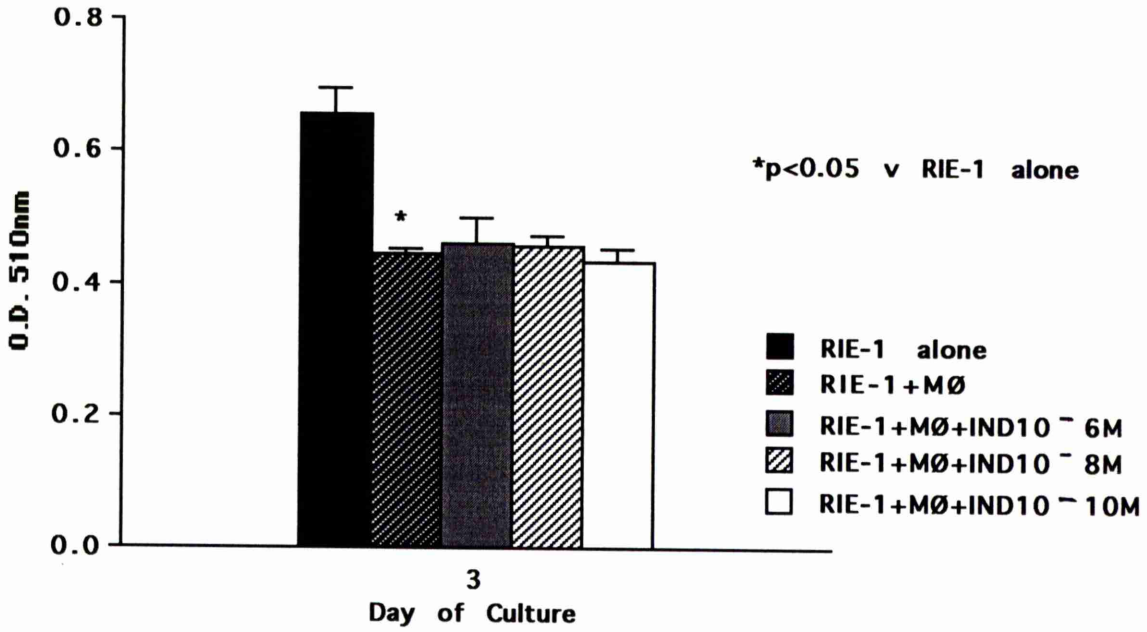

Fig 12b. The effect of indomethacin on the growth inhibition of RIE-1 cells by unstimulated macrophages as assessed by the MTT assay. Results shown are O.D./well+1s.e. for quadruplicate wells.

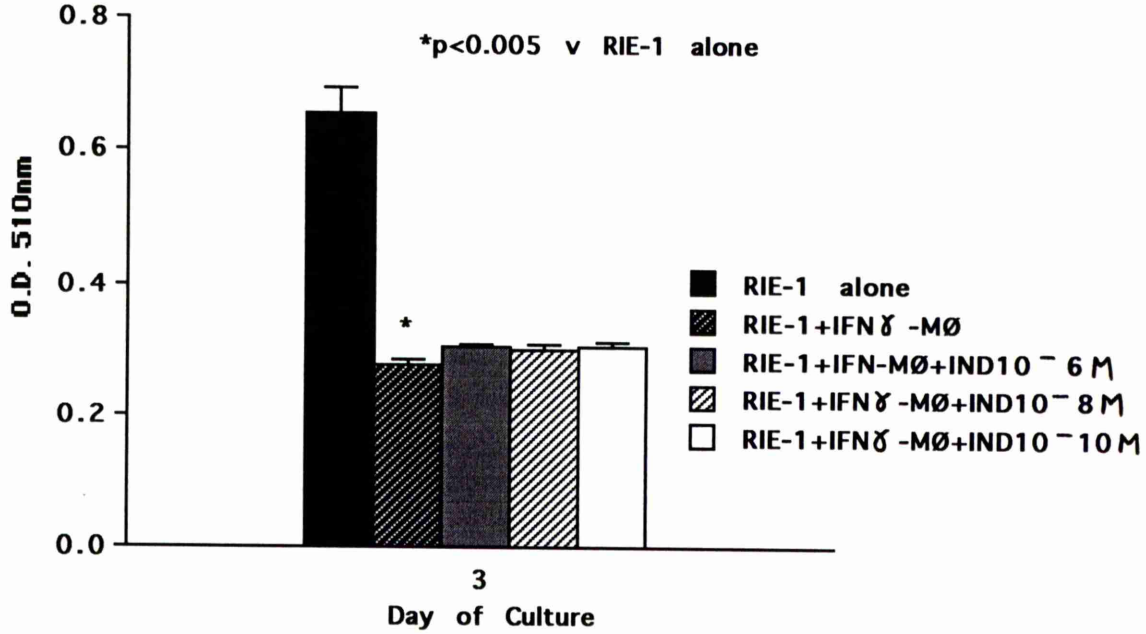

Fig 12c: The effect of indomethacin on the growth inhibition of RIE-1 cells by IFNγ activated macrophages as assessed by the MTT assay. Results shown are O.D./well+1s.e. for quadruplicate assays.

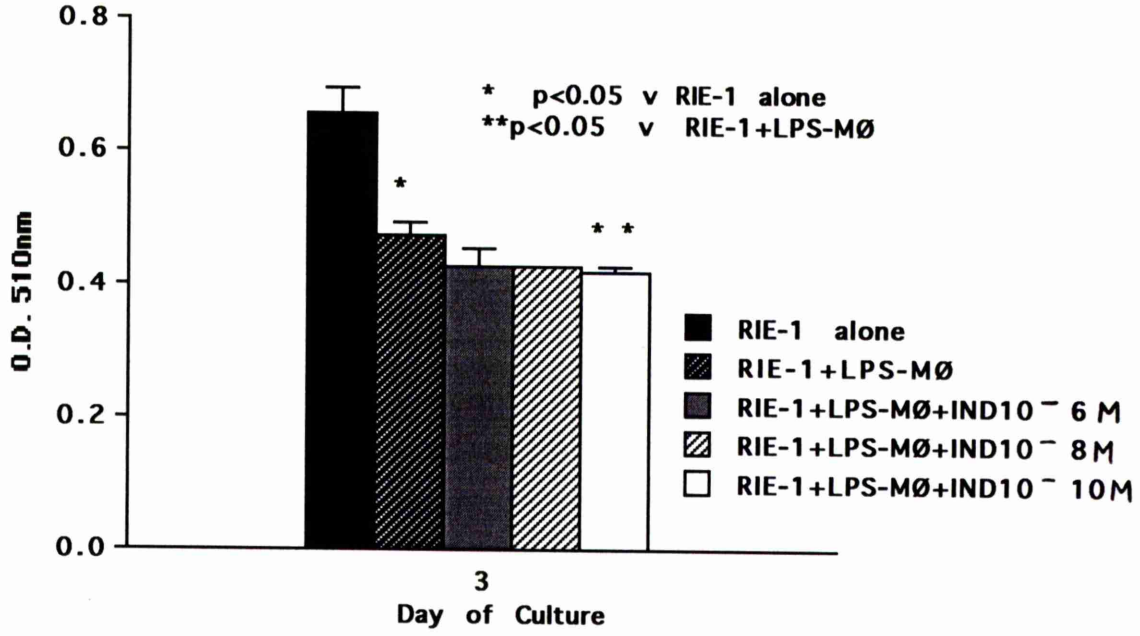

Fig 12d: The effect of indomethacin on the growth inhibition of RIE-1 cells by LPS activated macrophages as assessed by the MTT assay. Results shown are O.D./well+1s.e. for quadruplicate assays.

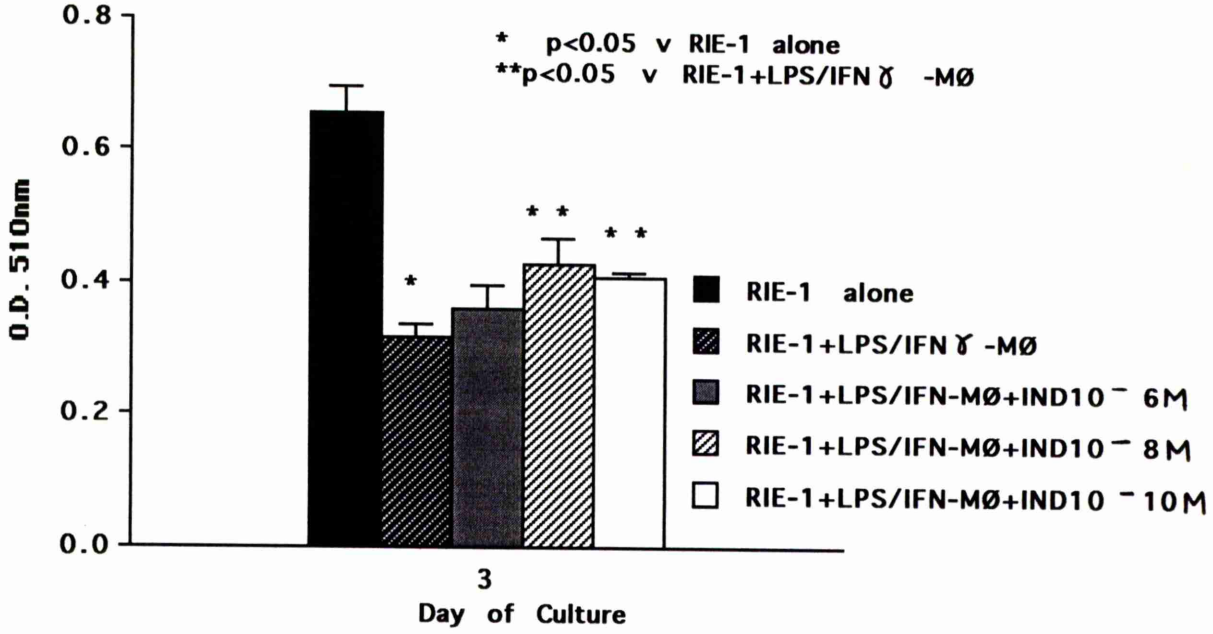

Fig 12e: The effects of indomethacin on growth inhibition of RIE-1 cells by LPS and IFNγactivated macrophages as assessed by the MTT assay. Results shown are O.D./well+1s.e. for quadruplicate assays.

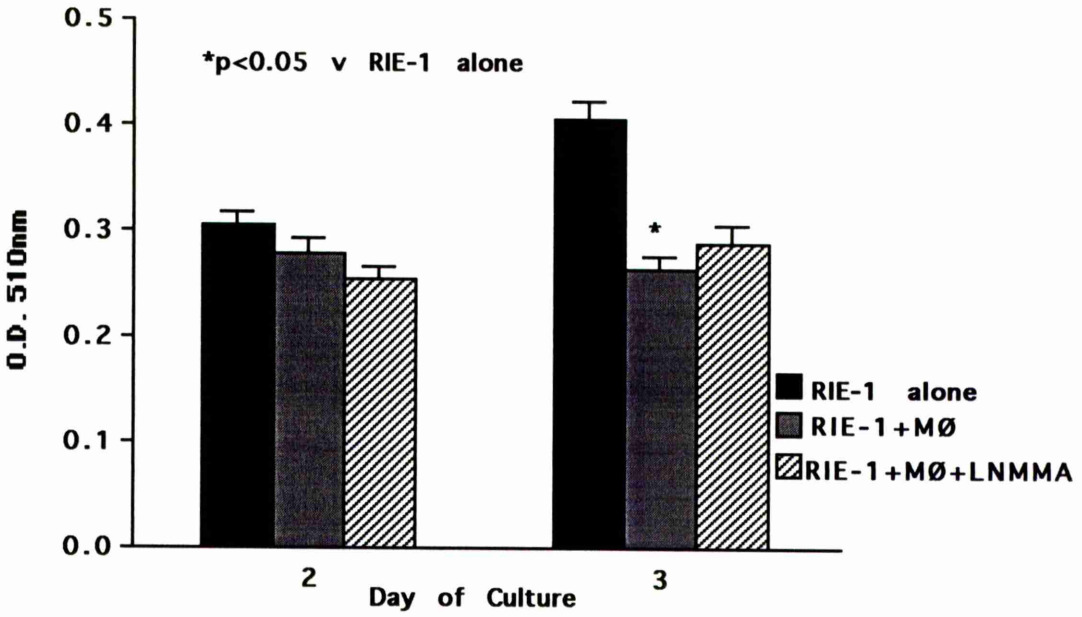

Fig 13a: The effect of L-NMMA on the growth inhibition of RIE cells by unstimulated macrophages as measured by the MTT assay. Results shown are O.D./well+1s.e. for quadruplicate assays.

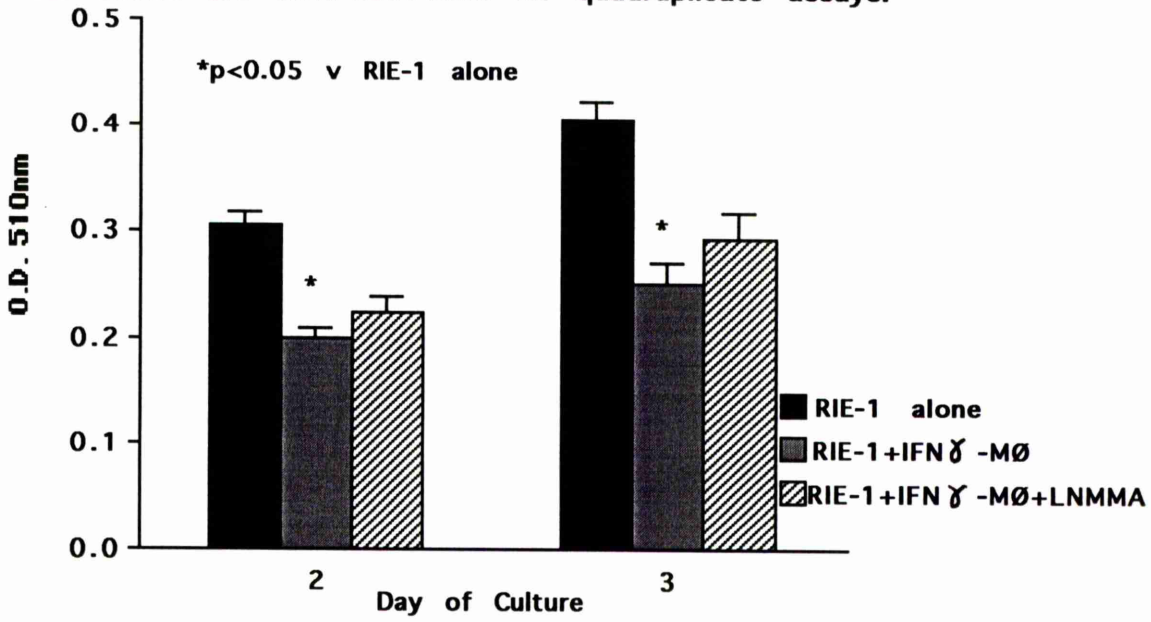

Fig 13b: The effect of L-NMMA on the growth inhibition of RIE-1 cells by IFNγ activated macrophages as assessed by the MTT assay. Results shown are O.D./well+1s.e. for quadruplicate assays.

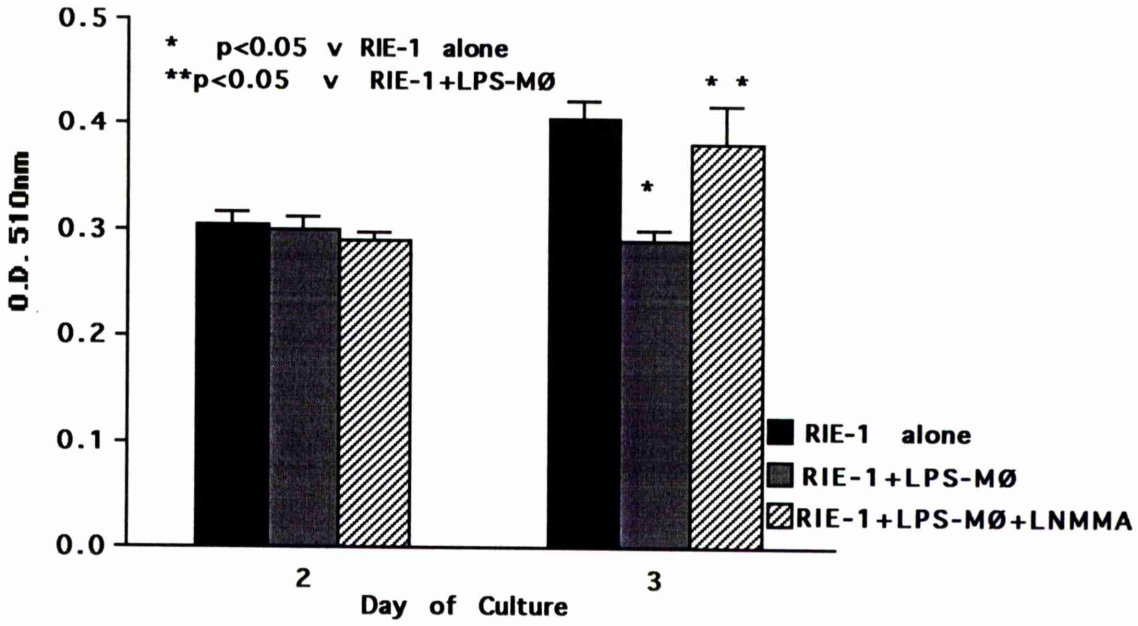

Fig 13c: The effect of L-NMMA on the growth inhibition of RIE-1 cells by LPS activated macrophages as assessed by the MTT assay. Results shown are O.D./well+1s.e. for quadruplicate assays.

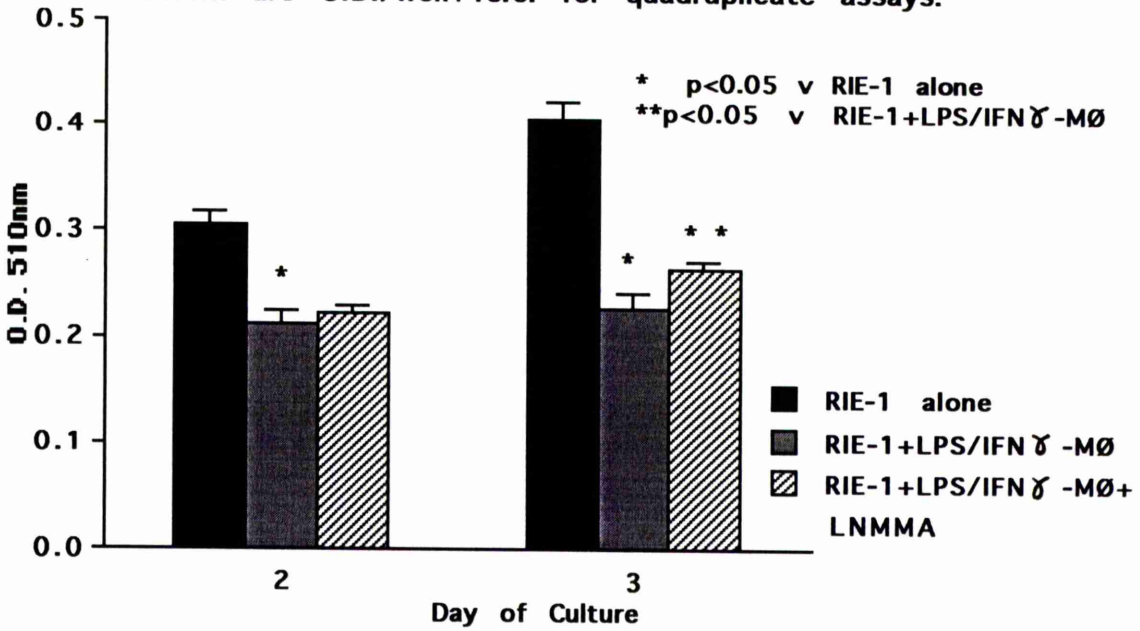

Fig 13d. The effect of L-NMMA on the growth inhibition of RIE-1 cells by LPS and IFNγ stimulated macrophages as assessed by the MTT assay. Results shown are O.D./well+1s.e. for quadruplicate assays.

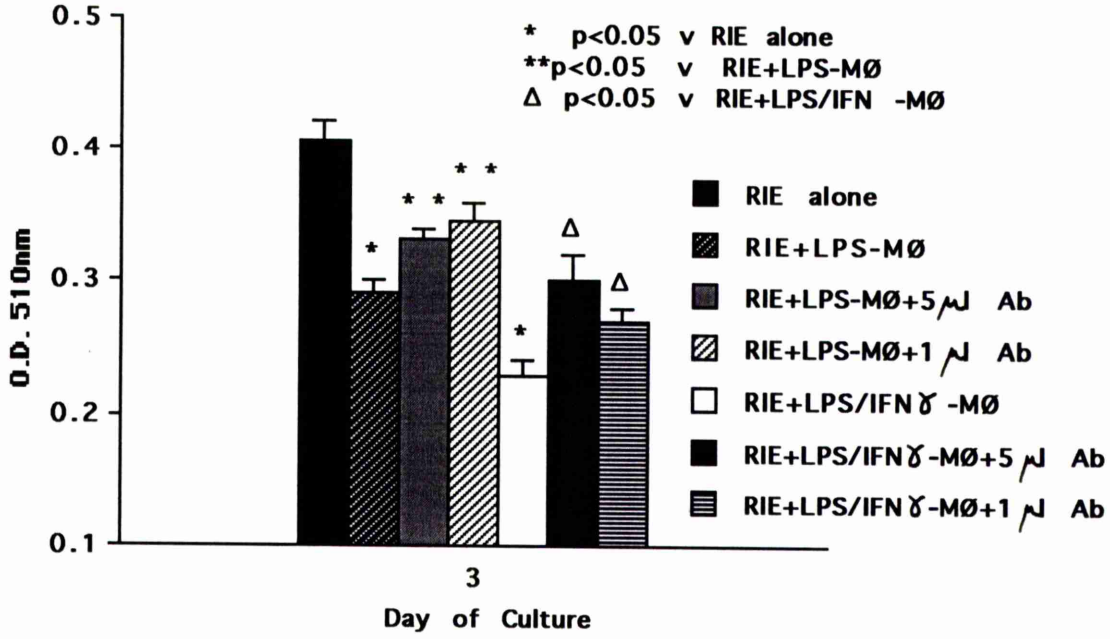

Fig 14a: The effect of an anti-TNFα antibody (E. Liew) on the growth inhibition of RIE-1 cells by activated macrophages as measured by the MTT assay. Results shown are O.D./well+1s.e. for quadruplicate assays.

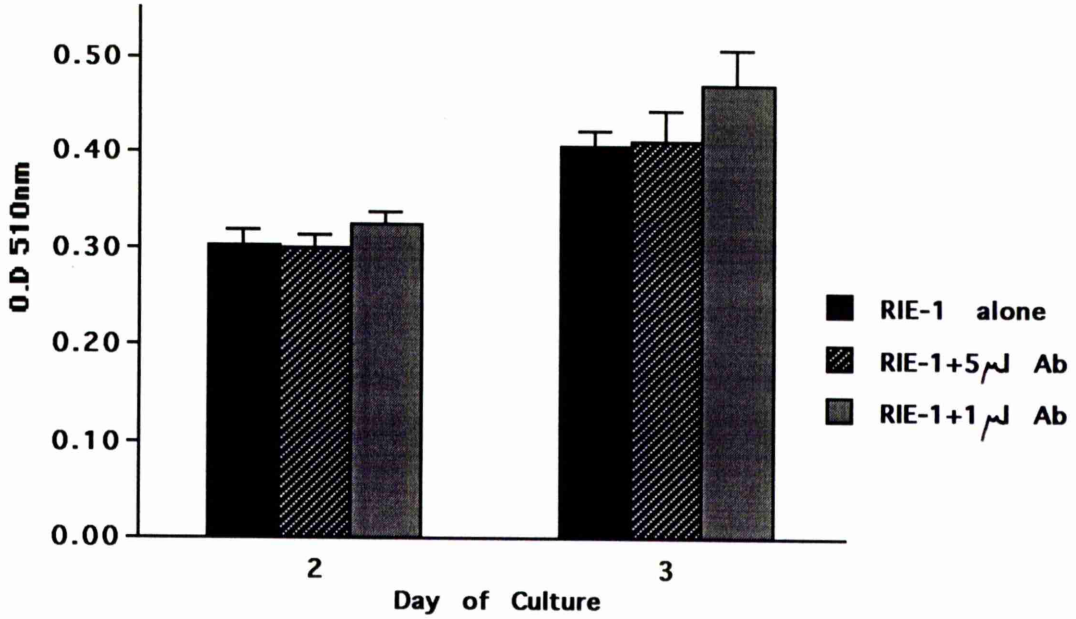

Fig 14b: The effect of polyclonal mouse anti-TNFα antibody on the growth of RIE-1 cells as measured by the MTT assay.
results shown are O.D./well+1s.e. for quadruplicate assays.

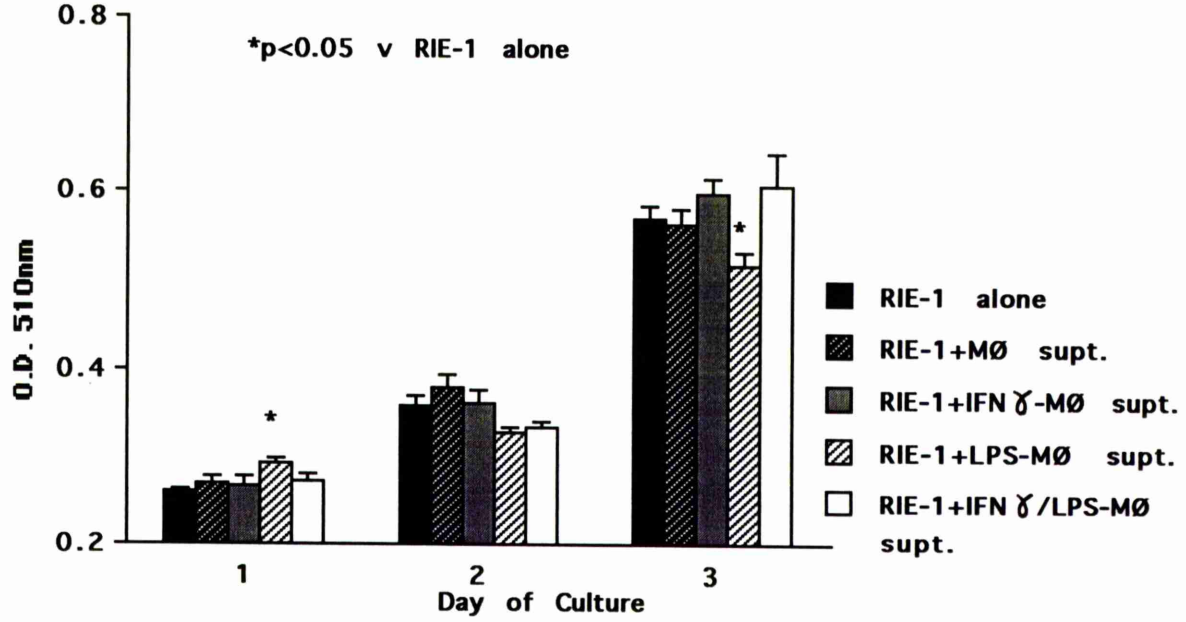

Fig 15a: The effect of day 1 macrophage supernatants on the growth of RIE-1 cells as measured by the MTT assay.
Results shown are O.D./well+1s.e. for quadruplicate assays.

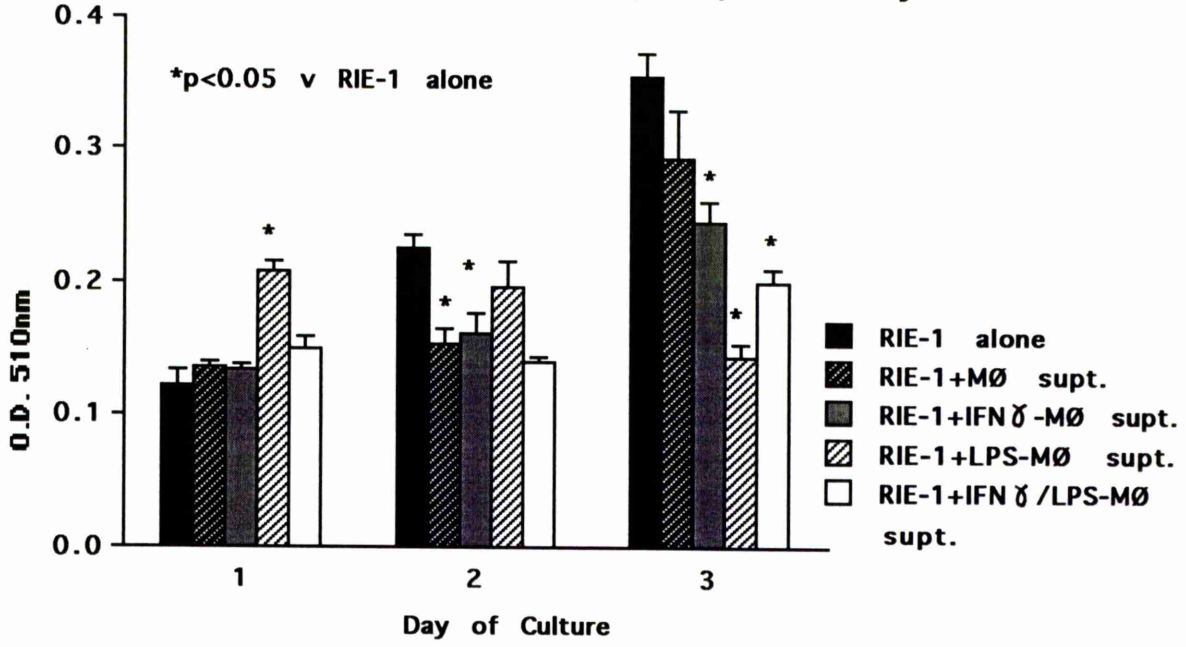

Fig 15b: The effect of day 2 macrophage supernatants on the growth of RIE-1 cells as measured by the MTT assay.
Results shown are O.D./well+1s.e. for quadruplicate assays.

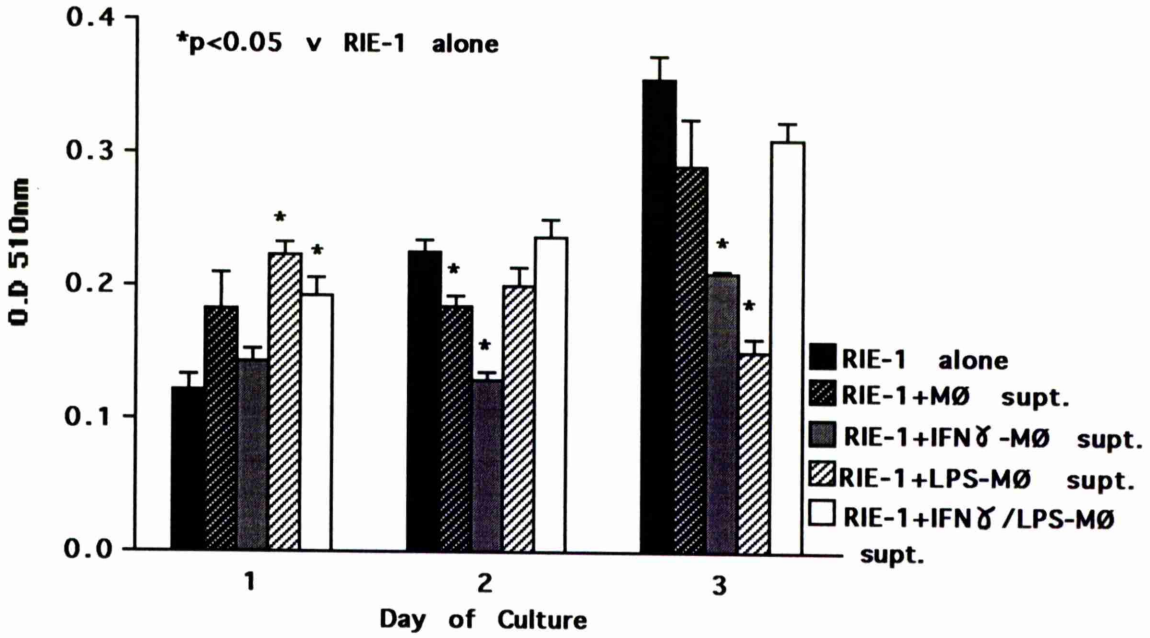

Fig 15c: The effect of day 3 macrophage supernatants on the growth of exponentially growing RIE-1 cells as measured by the MTT assay. Results shown as O.D./well+1s.e. for quadruplicate assays.

Target Cells	% Cytotoxicity
YAC-1 100:1	37
YAC-1 50:1	35
YAC-1 25:1	27
RIE-1 100:1	-2
RIE-1 50:1	-0.5
RIE-1 25:1	-0.5

Table 1: NK activity in rat spleen measured against YAC-1 and RIE-1 target cells. Results shown are % cytotoxicity at different E:T ratios.

CHAPTER 5
THE EFFECTS OF INDIVIDUAL CYTOKINES ON THE GROWTH OF RIE-1 CELLS

Introduction

In the previous chapter, I showed that activated lymphocytes and macrophages or their soluble products could influence the growth of an intestinal epithelial cell line *in vitro*. This is consistent with *in vivo* evidence that cytokines are involved in enteropathy, and here I went on to examine whether individual purified cytokines could reproduce any of the effects of lymphocytes and macrophages on the RIE-1 cell line.

For these studies, I chose those cytokines which might be present in T lymphocyte or macrophage supernatants and which had been implicated by my earlier experiments using blocking antibodies. I also examined additional mediators which have been suggested to be involved in clinical or experimental enteropathies such as intestinal GvHR, or which are thought to be involved in other forms of immunopathology. I investigated the effects of cytokines on both rapidly growing and confluent cultures in order to see if the stage of cell cycle altered the effect of a cytokine. It was thought that this might be important, as there are different zones of epithelial cells in the crypts, some of which contain proliferating cells and others which have ceased to divide.

Results

THE EFFECT OF CYTOKINES ON EXPONENTIALLY GROWING RIE-1 CELLS

In these experiments, RIE-1 cells were grown in the presence or absence of individual cytokines immediately after subculture. Unless otherwise stated, the experiments were carried out a minimum of 3 times and representative experiments are presented here.

Interferon γ

In the previous chapter I found that antibody to IFNγ partially reversed the cytostatic effect of supernatants from activated MLN cells on RIE-1 cells. In addition, anti-IFNγ antibody blocks intestinal GvHR *in vivo* (Mowat, 1988). Thus, I decided to examine whether IFNγ itself could have growth inhibitory effects. The effects of varying concentrations of recombinant rat IFNγ on the exponential growth of RIE-1 cells were measured by an MTT assay and are shown in Fig. 1. Control RIE-1 cells showed a steady increase in growth over the 4 day period. On day 1, IFNγ had no effect on the growth of the cells, except at a concentration of 100U/ml, which produced a barely significant inhibition of growth compared with control cells. However, by day 2 IFNγ had a consistent cytostatic effect on the growth of the RIE-1 cells, which was significant at all concentrations of IFNγ except 0.1U/ml. On days 3 and 4, IFNγ showed a dose dependent inhibitory effect on the growth of the RIE-1 cells and this was significant with even the lowest dose of IFNγ. Thus, IFNγ had a marked cytostatic effect on the growth of the RIE-1 cells, with high doses inhibiting growth by greater than 50%. Although IFNγ markedly inhibited the growth of the RIE-1 cells, it did not appear to have cytotoxic effects and the cells appeared healthy by light microscopy throughout the course of the experiment.

I then went on to investigate how IFNγ might interact with mediators thought to be important in physiological regulation of epithelial cell growth. First I examined the effect of IFNγ in the presence of EGF on epithelial cell growth. As described in chapter 1, 10ng/ml EGF had a stimulatory effect on the growth of the RIE-1 cells which was significant on days 3 and 4 of culture (Fig. 2). Conversely,

addition of IFNγ alone at a concentration of 10U/ml had a marked inhibitory effect on the growth of the RIE-1 cells, which was significant on days 3 and 4 of culture. Addition of IFNγ also reduced the stimulatory effect of the EGF, with growth now being virtually equivalent to that of control RIE-1 cells and being significantly lower than that found in the presence of EGF alone on days 2 and 4. However, with the exception of day 3, the levels were always significantly higher than with IFNγ alone. Thus, IFNγ and EGF have competing effects on the growth of the RIE-1 cells, with IFNγ markedly reducing the stimulatory effects of EGF.

Next, I investigated whether IFNγ could act in a synergistic manner with TGFβ, a potent inhibitor of epithelial cell growth. Fig3. shows the effect of 1U/ml IFNγ and 0.01ng/ml TGFβ on RIE-1 cell growth, doses which I had found previously to be sub-optimal. On day 1, neither TGFβ or IFNγ alone had any significant effect on the growth of the RIE-1 cells, but the two cytokines together had a significant inhibitory effect on the growth of the cells. On day 2, TGFβ alone still had no effect on growth, but by now IFNγ had a small but significant cytostatic effect and the two cytokines together had a marked cytostatic effect which was significantly greater than that caused by IFNγ alone. Thus, IFNγ and TGFβ have a synergistic cytostatic effect on RIE-1 cell growth. In a repeat experiment, this synergy was still present on day 3 of culture. Again, light microscopy indicated that the cells remained healthy after treatment with the cytokines, suggesting that the inhibition of growth was not due to cell death.

Tumour Necrosis Factor α

Studies have shown that depletion of TNFα *in vivo* can prevent enteropathy in intestinal GvHR and in chapter 2, I showed that an anti-TNFα antibody could partially reverse the cytostasis caused by activated macrophages. Therefore, in this experiment, I examined whether TNFα itself could have any effect on the growth of RIE-1 cells. On day 1 of culture, 1 or 10U/ml of TNFα had a small but significant stimulatory effect on the growth of the RIE-1 cells, but there was no significant effect at 0.1 and 100U/ml (Fig. 4). 0.01U/ml of TNFα had a cytostatic effect on the growth of the RIE-1 cells. However, upon repeating this experiment, I failed to reproduce either the growth stimulatory or inhibitory effects and, on day 2, no concentration of TNFα had any significant effect on the growth of the cells. On days 3 and 4, all concentrations of TNFα markedly inhibited the growth of the RIE-1 cells, although there was no clear dose dependent effect. Although TNFα is known to be cytotoxic to a number of cell types *in vitro*, light microscopy indicated that there was no cell death in any of the RIE-1 cell cultures treated with TNFα. Thus, I concluded that TNFα is inhibitory to the growth of RIE-1 cells in the exponential phase of growth, but that this inhibition takes greater than 48 hours to develop.

Interleukin 1

IL1 is another product of activated macrophages and is likely to be present in the macrophage supernatants used previously. In addition, recent experiments in our laboratory suggest that anti-IL1 antibody partially inhibits intestinal GvHR *in vivo*. Thus, in this experiment I examined the effect of recombinant human IL1β on the exponential growth of RIE-1 cells. On day 1, all concentrations of IL1 had a

significant stimulatory effect on the growth of the RIE-1 cells (Fig. 5). However, this was very small and no concentration of IL1 had any significant effect on the growth of the RIE-1 cells at other times of culture.

Interleukin 2

IL2 is a major product of activated T lymphocytes and is likely to have been a major constituent of the ConA activated MLN cell supernatants used previously. However, recombinant human IL2 at concentrations of 10-1000U/ml had no significant effect on the growth of the RIE-1 cells at any time over 4 days of culture (Fig. 6).

Interleukin 3

IL3 stimulates the growth and differentiation of progenitor cells from several tissues and is probably responsible for the hyperplasia of mucosal mast cells which occurs during intestinal GvHR. I therefore investigated the effect of IL3 on the growth of RIE-1 cells. Recombinant mouse IL3 at concentrations of 1-500U/ml had no significant effect on the growth of the RIE-1 cells at any time of culture (Fig. 7).

Interleukin 4

IL4 is another product of activated T lymphocytes and it has been suggested there may be IL4 receptors on epithelial cells including enterocytes. Recent work in this laboratory has also shown that intestinal GvHR in mice can be blocked with the soluble IL4 receptor and in this experiment I examined the effect of IL4 on the growth of RIE-1 cells. As purified or recombinant rat IL4 was not available, I

used a supernatant derived from a CHO cell line transfected with the rat IL4 gene. On day 1, there was no significant effect of the IL4 containing supernatant on the growth of the RIE-1 cells (Fig. 8). However, on day 2, both 1% and 5% of the IL4 supernatant significantly increased growth compared with controls. These effects were not apparent on day 3, but on day 4, the supernatant had a clear dose dependent ability to increase the growth of the RIE-1 cells. Although these results suggest that IL4 may stimulate the growth of RIE-1 cells *in vitro*, I was unable to confirm that IL4 itself was responsible for the effect on growth, as I did not have a control supernatant from a non-transfected cell line and no anti-rat IL4 blocking antibody was available.

Interleukin 6

IL6 is another product of activated macrophages and T lymphocytes. In addition, intestinal epithelial cells produce IL6 and have surface receptors for IL6. As a result, it has been suggested that IL6 may act as an autocrine regulator of epithelial cell growth (Shirota et al., 1990). However, 1-500U/ml of recombinant human IL6 had no significant effect on the growth of RIE-1 cells (Fig. 9). The apparent inhibition of growth on day 4 using 50U/ml IL6 is probably an anomaly, although I was unable to repeat this experiment due to lack of the cytokine.

Interferon α/β

IFNα/β is another product of activated macrophages and has been shown to cause enteropathy when administered to normal mice *in vivo*. Fig. 10 shows that 0.1-100U/ml of purified mouse IFN α/β had

no significant effect on the growth of the RIE-1 cells on any of the 3 days tested, except on day 1, where 100U/ml caused a significant increase in growth and on day 3 when 0.1U/ml produced some growth inhibition compared with controls. As repetition of this experiment showed no significant effects of these values of IFN α/β, I concluded that these isolated findings were unlikely to be of significance.

Nitric Oxide

In the previous chapter, I showed that blocking the production of NO partially inhibited some of the effects of macrophages on RIE-1 cells. Here, I investigated the effect of NO itself on the growth of the RIE-1 cells, by the addition of SNAP, which releases NO when placed into aqueous solution. On day 1, the two highest concentrations of SNAP had a significant inhibitory effect on the growth of the RIE-1 cells, although the other concentrations had no significant effect (Fig. 11). On day 2, most concentrations of SNAP were also cytostatic, but the pattern was different to that found on day 1 and on day 3 SNAP showed an inhibitory effect on growth only at the highest concentration. Unfortunately, time did not permit me to investigate whether these rather inconsistent effects reflected a true ability of NO to inhibit RIE-1 cell growth.

EFFECT OF CYTOKINES ON CONFLUENT CULTURES OF RIE-1 CELLS

As described in the introduction, the crypt contains cells in different stages of the cell cycle. In the next series of experiments, I looked at the effects of cytokines on confluent cultures of RIE-1 cells, in which the basal rate of cell turnover is low. The aim of this was twofold.

First, stimulatory effects could have been masked in the exponential cultures used previously, as the cells were already turning over rapidly. Secondly, the action of cytokines which act by stimulating resting cells to divide would only be revealed in cultures of resting cells. Cultures of RIE-1 cells were seeded 3 days prior to the experiment so that the cells were confluent upon addition of the cytokines. Again, experiments were carried out at least 3 times unless otherwise stated and representative experiments are presented here.

Transforming Growth Factor β

As TGFβ has been implicated as an autocrine regulator of epithelial cell growth and is also a product of macrophages, I initially examined the effects of TGFβ on the growth of confluent RIE-1 cells. In contrast to the potent cytostatic effects on growing RIE-1 cells described in chapter 1, no concentration of purified TGFβ1 had any significant effect on the growth of the confluent RIE-1 cells on any of the days assayed (Fig. 12). Thus, TGFβ only inhibits the growth of RIE-1 cells in the exponential phase of growth.

Interferon γ

As with exponentially growing cells, recombinant rat IFNγ caused a marked dose dependent decrease in growth at all times of culture (Fig. 13). In addition, the number of cells in IFNγ treated cultures were much lower than day 1 controls, suggesting that IFNγ was causing cell death. However, this was not obvious from light microscopy where the cells appeared healthy.

Tumour Necrosis Factor α

The highest concentration of recombinant human TNFα (1000U/ml) stimulated the growth of confluent RIE-1 cells at all times examined, although other concentrations of TNFα had no consistent effect except for a small but significant stimulatory effect of 100U/ml on day 2 (Fig. 14). Repeat experiments confirmed that 100 and 1000U/ml of TNFα had a consistent stimulatory effect on the growth of confluent cultures of RIE-1 cells. These results contrast with the inhibitory effects of TNFα on exponentially growing cultures of RIE-1 cells.

Interleukin 1

The effect of recombinant mouse IL1β on confluent cultures of RIE-1 cells was examined next. Addition of this cytokine produced a clear dose dependent increase in growth which was very marked at higher concentrations (Fig. 15). Again, this contrasts with the absence of any effect of this cytokine on exponentially growing RIE-1 cells.

Interleukin 2

Fig. 16 shows that recombinant human IL2 had no significant effect on the growth of confluent RIE-1 cells on any of the days assayed. This finding is the same as that found with exponentially growing RIE-1 cells.

Interleukin 3

No concentration of recombinant mouse IL3 had any significant effect on the growth of confluent RIE-1 cells on either of the days assayed (Fig. 17). Thus, IL3 has no effect on the growth of either confluent or exponentially growing RIE-1 cells.

Interferon α/β

Fig. 18 shows that as with exponentially growing cells, purified mouse IFNα/β had no significant effect on the growth of confluent RIE-1 cells on either of the days assayed.

Conclusions

In this chapter I established that individual cytokines could have direct effects on the growth of RIE-1 cells and that these effects were dependent on whether the cells were in the exponential phase of growth or confluent.

IFNγ inhibited the growth of the RIE-1 cells in both the exponential and confluent stages of growth. The effects of IFNγ in the former were very potent, as it was inhibitory even in the presence of EGF. At sub-optimal doses it also synergised with TGFβ to increase growth inhibition. These results are consistent with my previous findings that IFNγ was partially responsible for the cytostatic effects of activated MLN cell supernatants and with *in vivo* work implicating IFNγ in enteropathy.

An IL4 containing supernatant had a stimulatory effect on the growth of the RIE-1 cells in the exponential phase of growth. Due to a limited availability of this cytokine I was unable to repeat this experiment using confluent cultures and I was also unable to prove that IL4 itself was responsible for this stimulatory effect, or whether it was another mediator present in the supernatant. However, my results suggest that IL4 may stimulate the growth of RIE-1 cells in the exponential phase of growth.

IL2, IFNα/β and IL3 had no effect on the growth of the RIE-1 cells either in the exponential phase of growth or when confluent. This is consistent with the fact that receptors for these cytokines on epithelial cells have never been reported, but it should be noted that I was unable to obtain rat derived sources of cytokines. It would be imoprtant to ensure that the lack of effect in my system did not reflect a lack of biological activity of the cytokines in the rat.

TNFα had opposite effects on the growth of RIE-1 cells depending on the stage of growth. Its inhibitory effect on the growth of cells in the exponential phase of growth is consistent with the inhibitory effects of TNFα on a number of other cell types *in vitro*. However, I did not find that TNFα was cytotoxic to normal RIE-1 cells in contrast to findings in other systems. Conversely, TNFα stimulated the growth of confluent RIE-1 cells, although this was only apparent at high concentrations of TNFα which seem unlikely to appear *in vivo*. These results support the findings in chapter 4, that TNFα may have been partly responsible for the activity of activated macrophages to inhibit the growth of actively growing RIE-1 cells. However, they also indicate that TNFα may stimulate resting RIE-1 cells to enter the cell cycle and divide.

IL1 had no effect on the growth of exponentially growing RIE-1 cells, but stimulated the growth of confluent cultures even at low doses of the cytokine. It is possible that this stimulatory effect may have been masked in exponential growth cultures where the cells would already be rapidly dividing. The efficacy of low doses of IL1 *in vitro*, suggest that this cytokine may also be important *in vivo*, possibly by stimulating the crypt cells which have stopped dividing.

IL6 also had no effect on the growth of RIE-1 cells in the exponential phase of growth, although epithelial cells have been shown to express receptors for this cytokine and IL6 has been implicated in growth regulation of enterocytes. However, human IL6 was used in my experiments and again it is possible that the cytokine is not reactive with rat IL6 receptors.

SNAP was used in these experiments as a source of NO. High doses appeared to have an inhibitory effect on the growth of RIE-1 cells in exponential phase of growth. This is in agreement with previous experiments in which the inhibitory effect of activated macrophage populations could be partially reversed by blocking NO synthesis by L-NMMA. That NO itself can have inhibitory effects on the growth of RIE-1 cells supports the view that it may be an important mediator in enteropathy.

Finally, I examined the effects of TGFβ on confluent cultures of RIE-1 cells. Previously, I showed that TGFβ inhibited the growth of RIE-1 cells in the exponential phase of growth. However, TGFβ had no effect on the growth of confluent cultures of RIE-1 cells. This suggests that TGFβ exerts its inhibition by acting on dividing cells and that it is not cytotoxic to the RIE-1 cells.

Thus cytokines can have direct effects on the growth of RIE-1 cells *in vitro* and the effects of a number of cytokines may differ depending on the stage of growth. However, most of these effects are inhibitory to growth which is contrary to the hypothesis that during enteropathy crypt hyperplasia is caused by a direct stimulatory effect of cytokines on crypt epithelial cells.

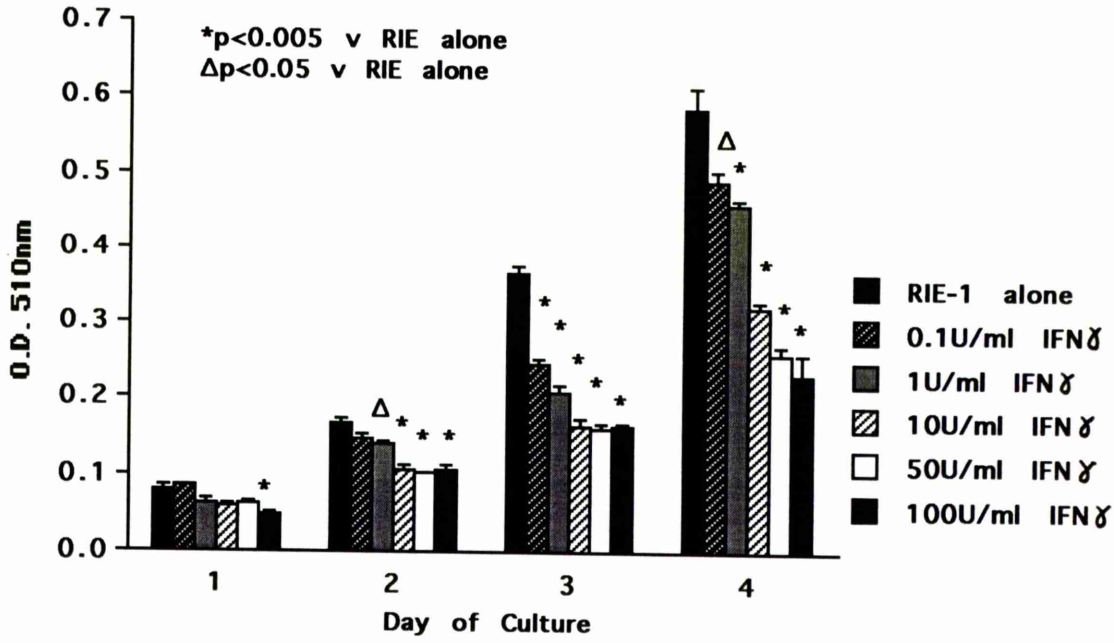

Fig 1: The effect of IFNγ on the growth of exponentially growing RIE-1 cells as measured by the MTT assay.
Results shown are mean O.D./well+1s.e. for quadruplicate assays.

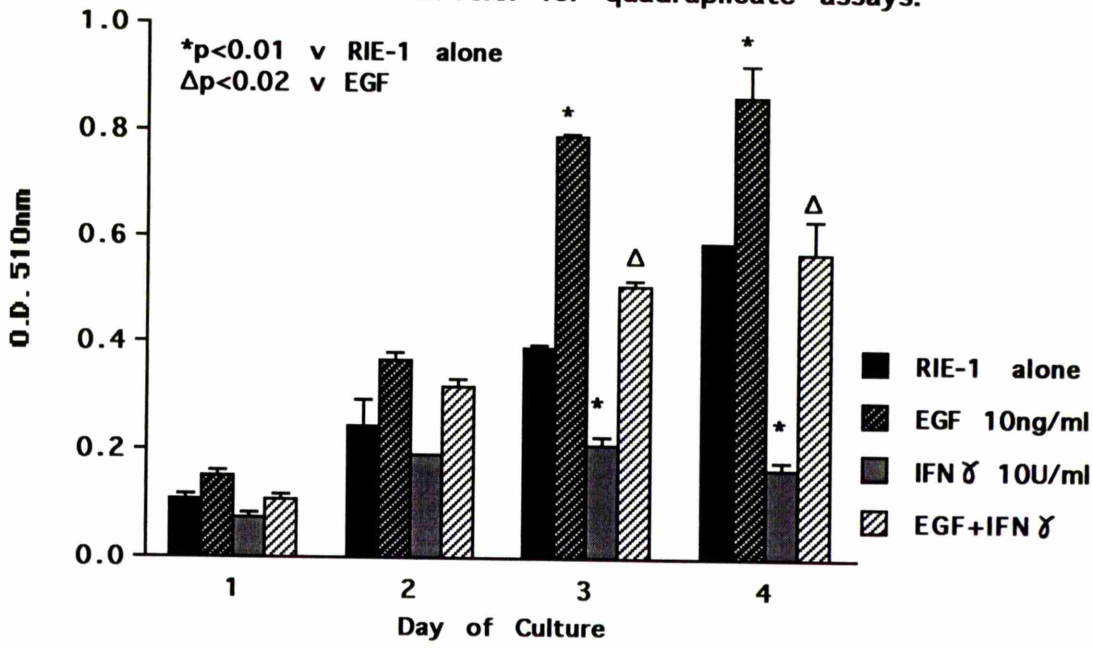

Fig 2: The effect of EGF(10ng/ml) and IFNγ(10U/ml) on the growth of exponentially growing RIE-1 cells as measured by the MTT assay.
Results shown are mean O.D./well+1s.e. for quadruplicate assays.

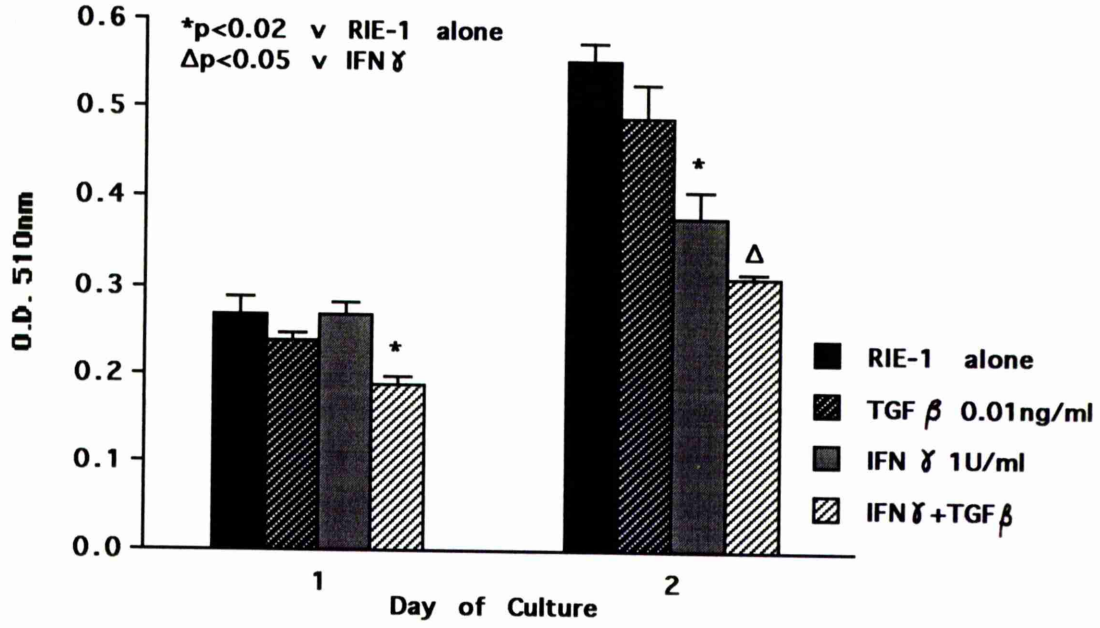

Fig 3: The effect of IFNγ 1U/ml and TGFβ 0.01ng/ml on the growth of exponentially growing RIE-1 cells as measured by the MTT assay.
Results shown are mean O.D./well+1s.e. for quadruplicate assays.

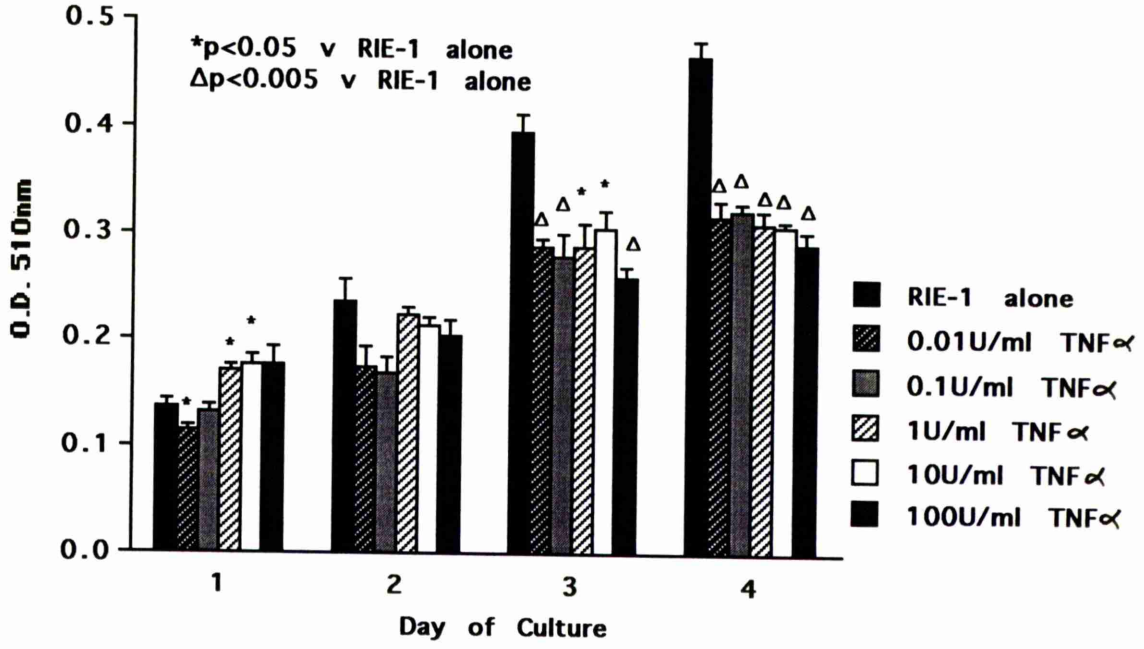

Fig 4: The effect of TNFα on the growth of exponentially growing RIE-1 cells as measured by the MTT assay.
Results shown are mean O.D./well+1s.e. for quadruplicate assays.

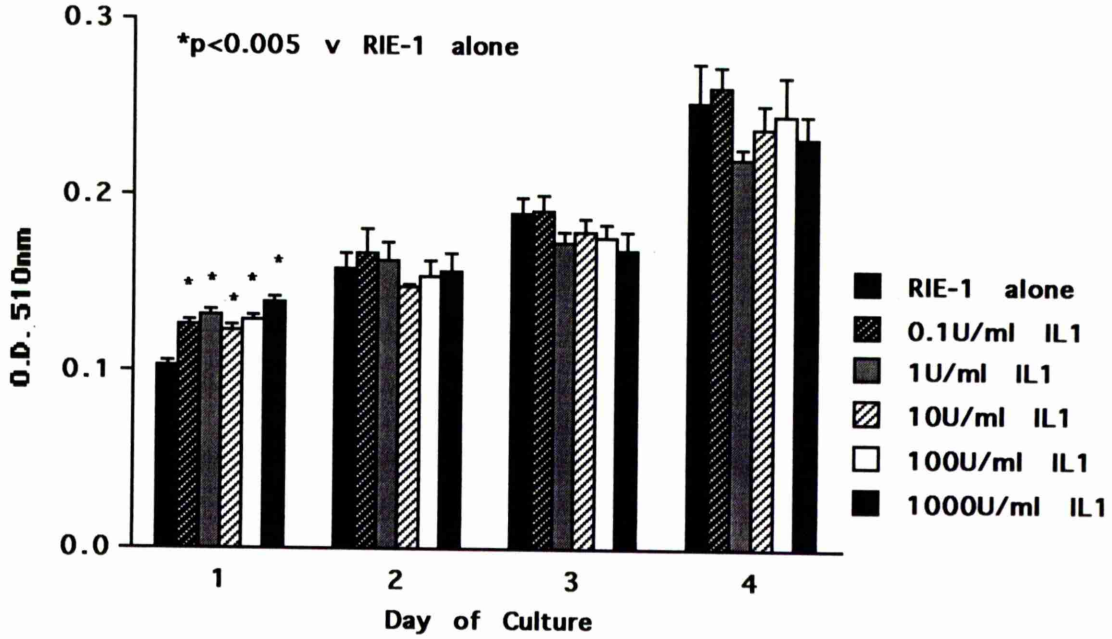

Fig 5: The effect of IL1 on the growth of exponentially growing RIE-1 cells as measured by the MTT assay.
Results shown are mean O.D./well+1s.e. for quadruplicate assays.

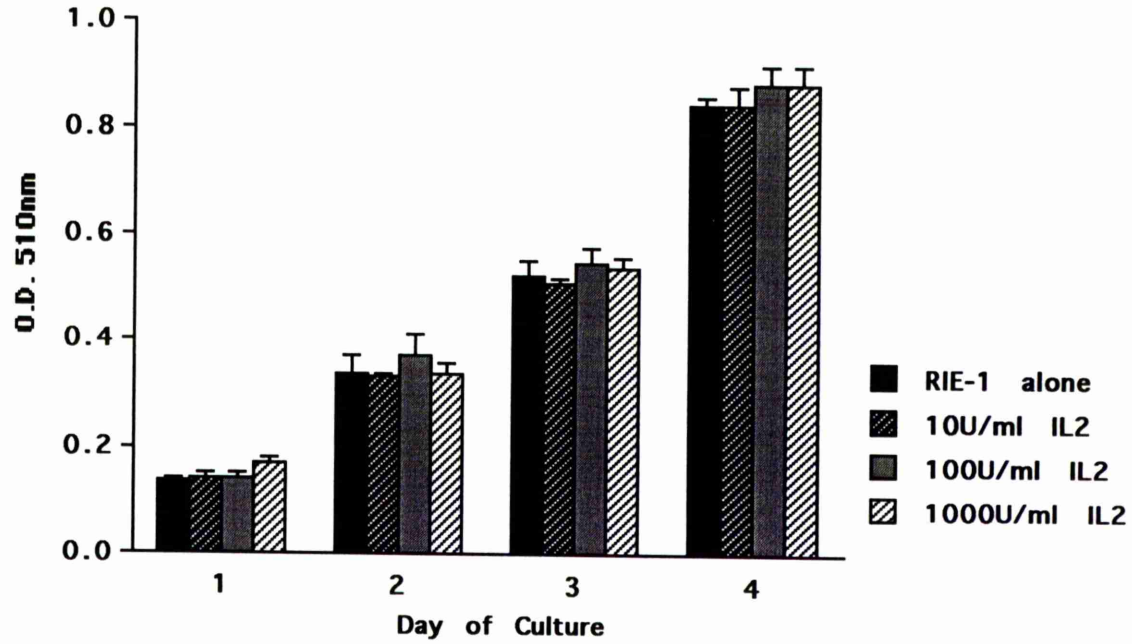

Fig 6: The effect of IL2 on the growth of exponentially growing RIE-1 cells as measured by the MTT assay.
Results shown are mean O.D./well+1s.e for quadruplicate assays.

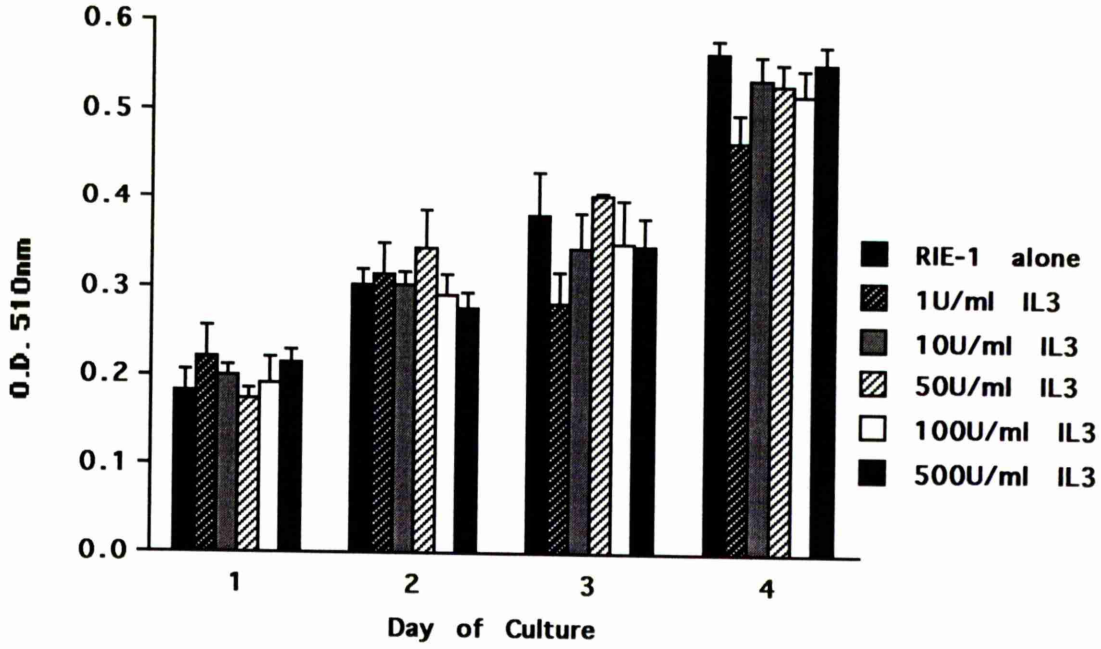

Fig 7: The effect of IL3 on the growth of exponentially growing RIE-1 cells as measured by the MTT assay.
Results shown are mean O.D./well+1s.e. for quadruplicate assays.

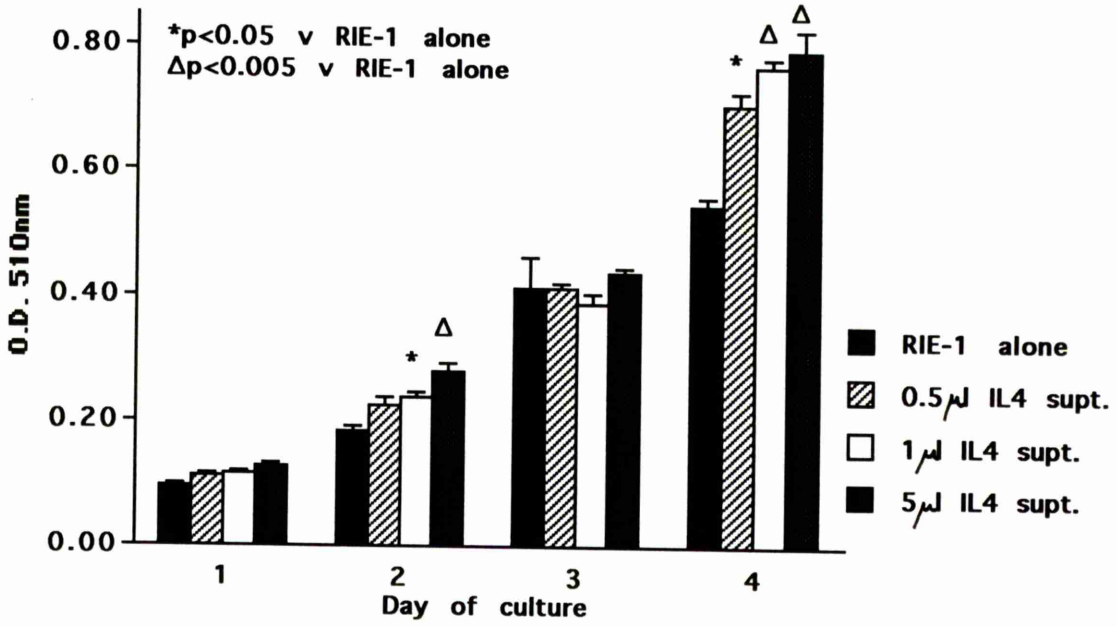

Fig 8: The effect of supernatant from an IL4 transfected CHO cell line on the growth of exponentially growing RIE-1 cells as measured by the MTT assay. Results shown are mean O.D./well+1s.e. for quadruplicate assays.

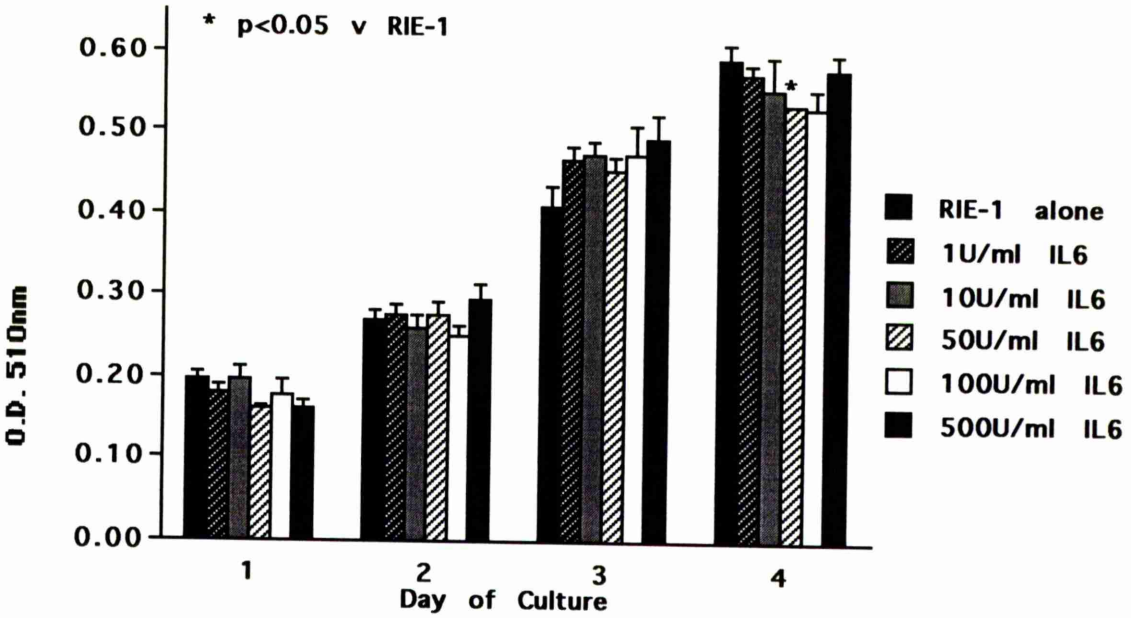

Fig 9: The effect of IL6 on the growth of exponentially growing RIE-1 cells as measured by the MTT assay.
Results shown are mean O.D./well+1s.e. for quadruplicate assays.

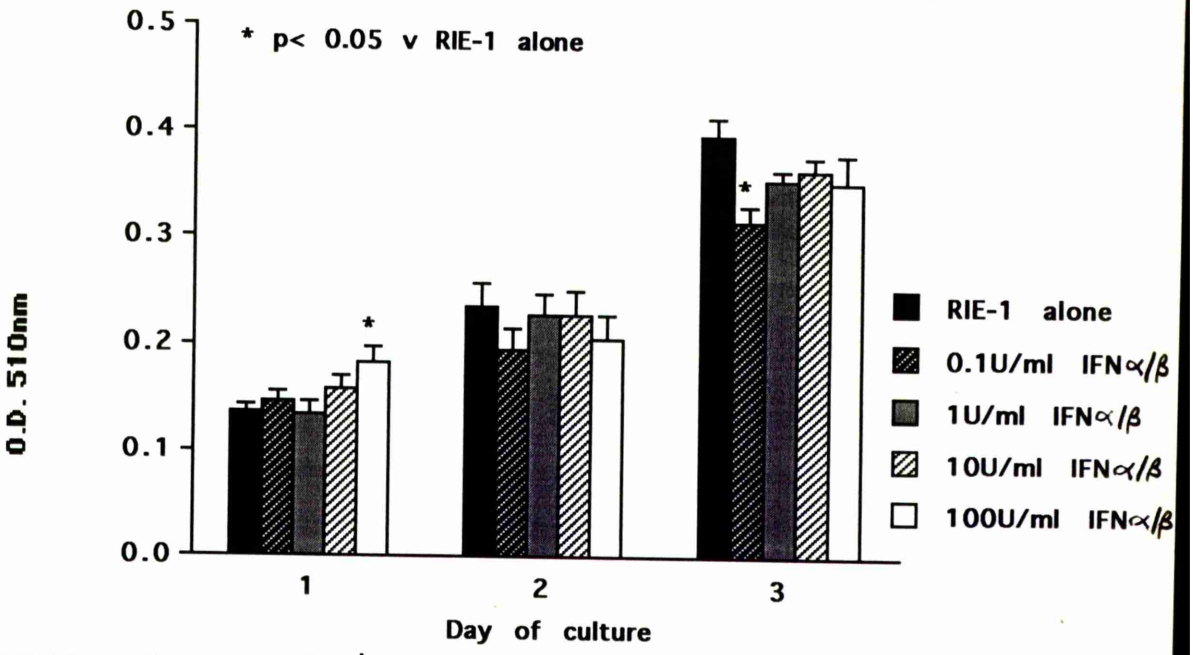

Fig. 10 The effect of IFN α/β on the growth of exponentially growing RIE-1 cells as assessed by the MTT assay.
Results shown are O.D/well +1s.e for quadruplicate assays.

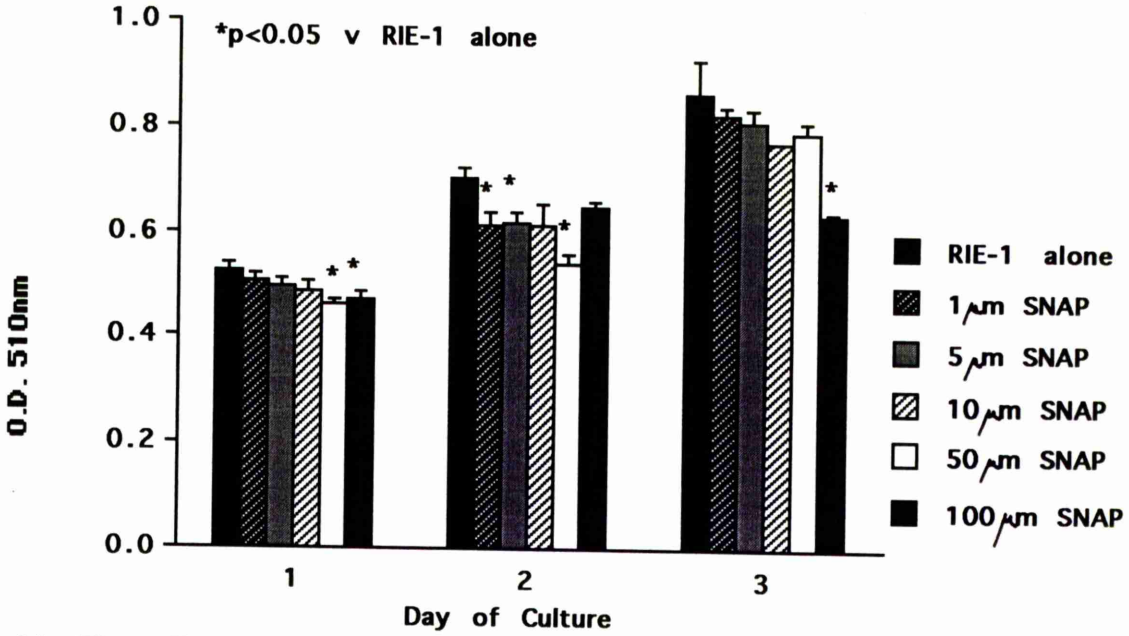

Fig 11: The effect of SNAP on the growth of exponentially growing RIE-1 cells as measured by the MTT assay.
Results shown are mean O.D./well+1s.e. for quadruplicate assays.

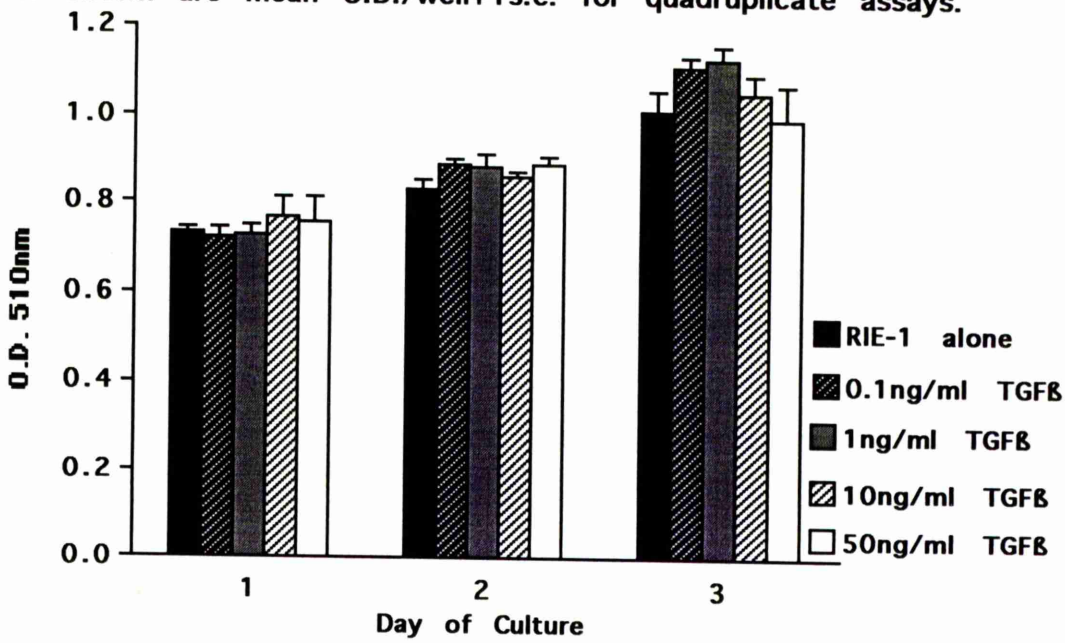

Fig 12: The effect of TGFß on the growth of confluent cultures of RIE-1 cells as measured by the MTT assay.
Results shown are mean O.D./well+1s.e. for quadruplicate assays.

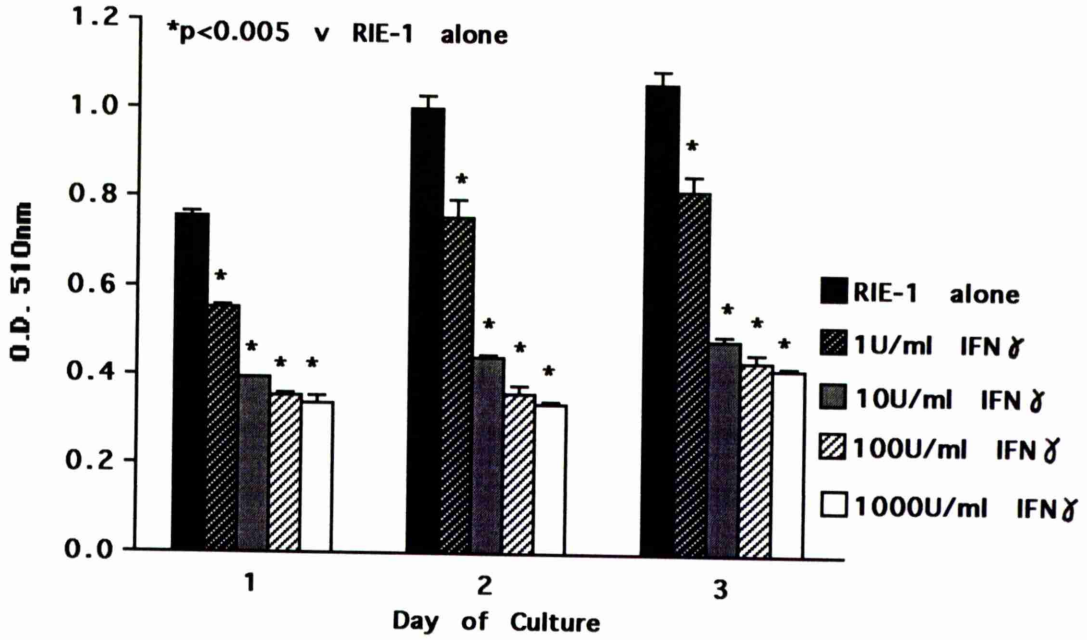

Fig 13: The effect of IFNγ on the growth of confluent cultures of RIE-1 cells as measured by the MTT assay. Results shown are mean O.D./well+1s.e. for quadruplicate assays.

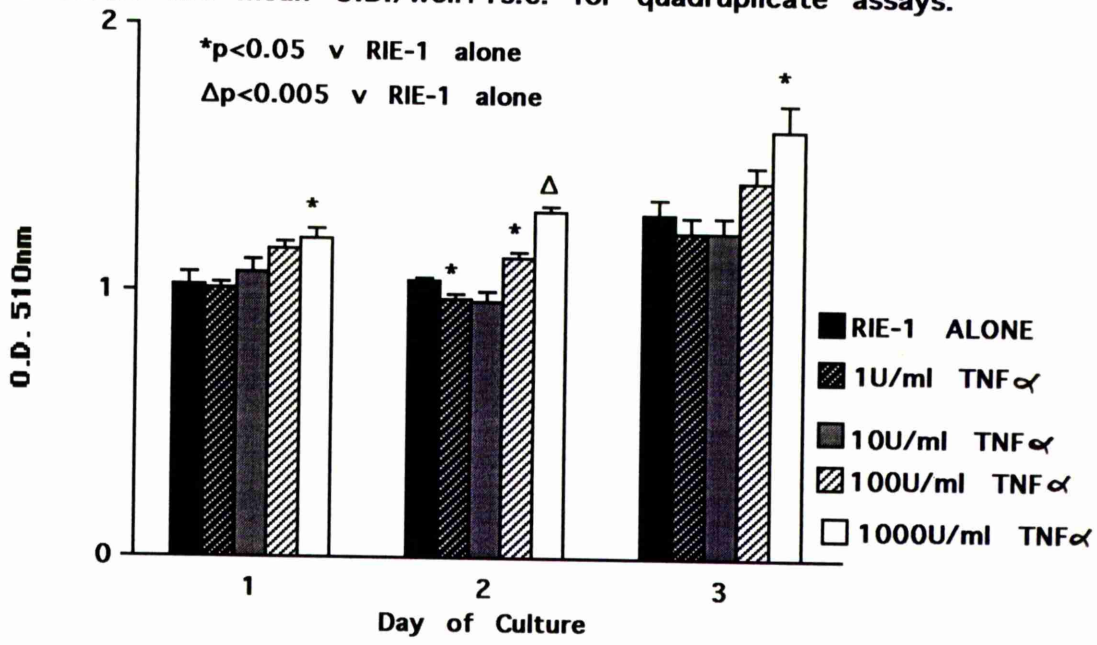

Fig 14: The effect of TNFα on the growth of confluent cultures of RIE-1 cells as measured by the MTT assay. Results shown are mean O.D./well+1s.e. for quadruplicate assays.

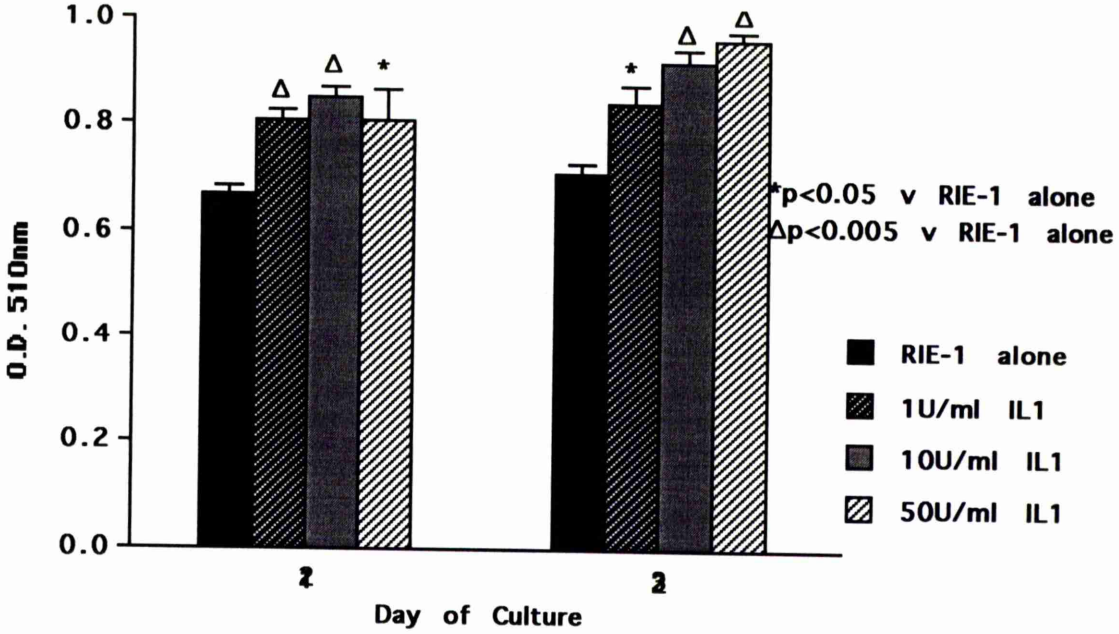

Fig 15: The effect of IL1 on the growth of confluent cultures of RIE-1 cells as measured by the MTT assay. Results shown are mean O.D./well+1s.e. for quadruplicate assays.

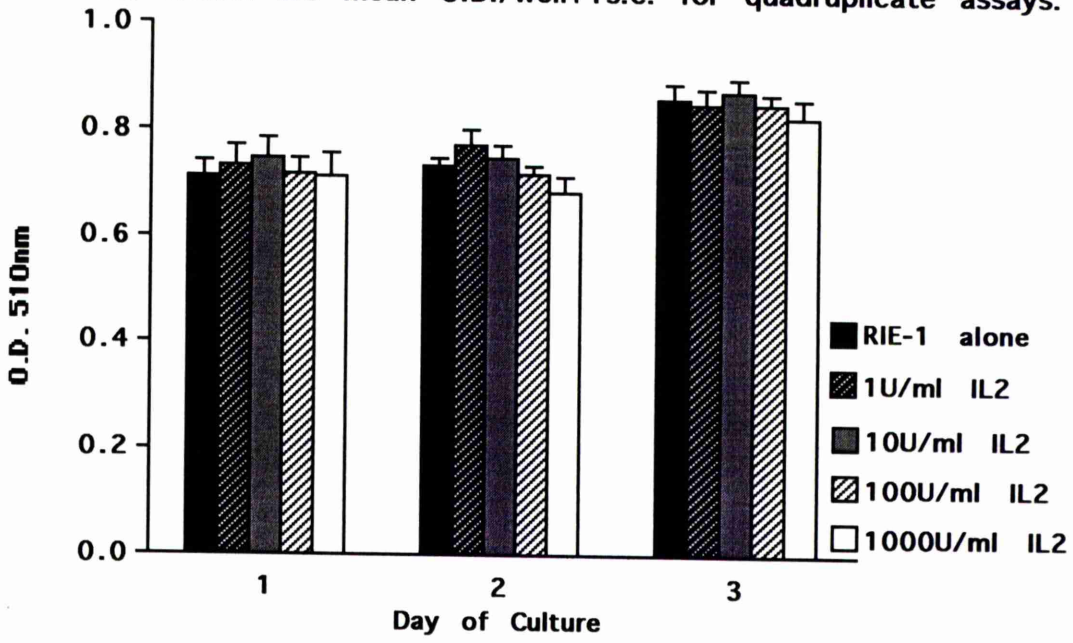

Fig 16: The effect of IL2 on the growth of confluent cultures of RIE-1 cells as measured by the MTT assay. Results shown are mean O.D./well+1s.e. for quadruplicate assays.

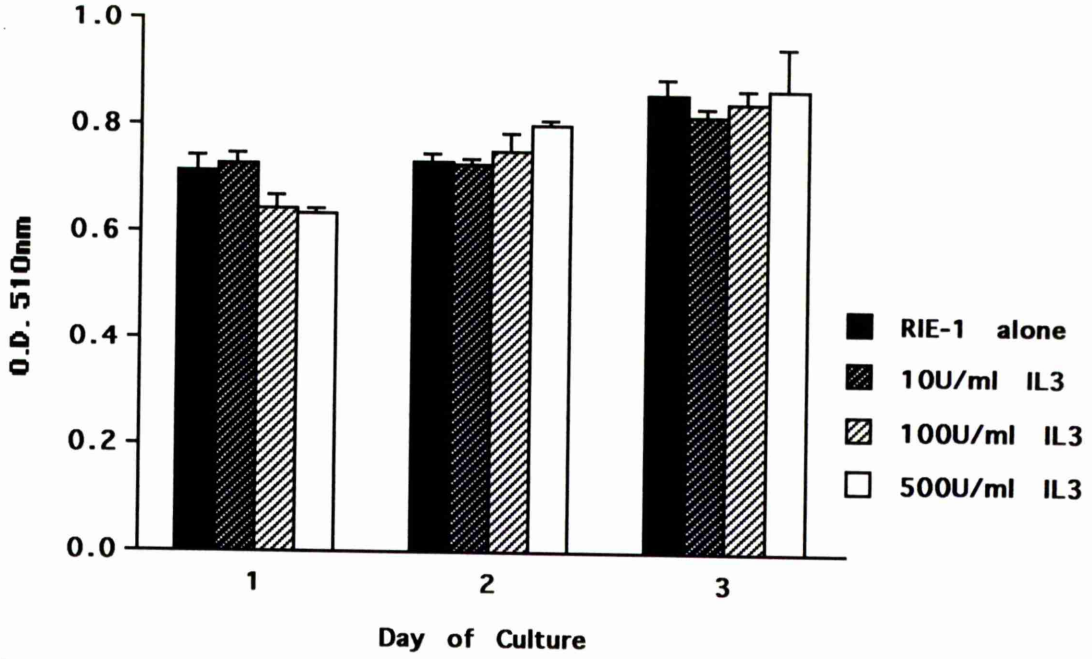

Fig 17: The effect of IL3 on the growth of confluent cultures of RIE-1 cells as measured by the MTT assay.
Results shown are mean O.D./well+1s.e. for quadruplicate assays.

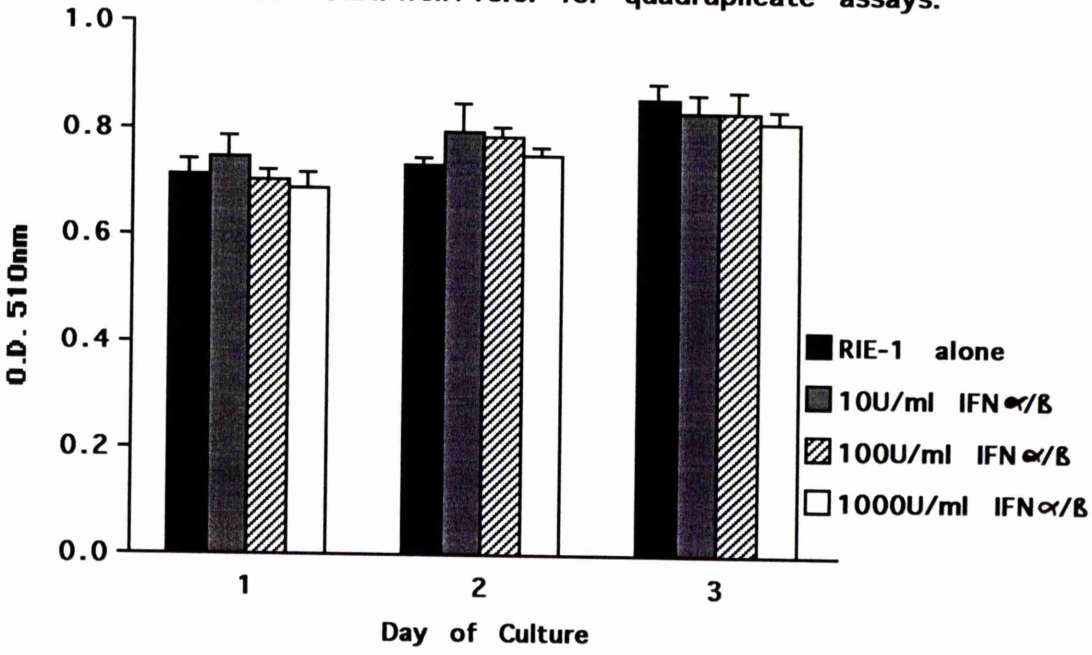

Fig 18: The effect of IFNα/ß on the growth of confluent cultures of RIE-1 cells as measured by the MTT assay.
Results shown are mean O.D./well+1s.e. for quadruplicate assays.

CHAPTER 6
THE EFFECTS OF CYTOKINES ON THE DIFFERENTIATION OF RIE-1 CELLS

Introduction

In previous chapters, I showed that certain cytokines had direct effects on the growth of RIE-1 cells. Although altered crypt cell proliferation is a major feature of enteropathy *in vivo*, these disorders are also associated with an increased rate and altered pattern of epithelial cell differentiation. Therefore, in this chapter I examined whether cytokines could influence the differentiation of the immature RIE-1 cells *in vitro*. As indices of differentiation, I measured total protein synthesis, before assessing the effects of IFNγ and TNFα on the expression of one particular group of proteins of pathological interest *in vivo*, the MHC antigens. I then looked for ultrastructural evidence of differentiation by electron microscopy and, finally, investigated whether cytokines could affect the ionic permeability of an RIE-1 cell monolayer.

Results

The Effects of Cytokines on Total Protein Synthesis of RIE-1 Cells

In this experiment, I looked at the effects of cytokines on the synthesis of proteins by RIE-1 cells, using cytokines which I had found to affect the growth of the RIE-1 cells. The synthesis of proteins was measured by assessing the uptake of ^{3}H leucine in cultures of RIE-1 cells. To allow for any effects of cytokines on cell number, RIE-1 cells were first grown to confluence and ^{3}H leucine uptake was corrected to a standard cell number.

Fig. 1 shows the effects of IFNγ on protein synthesis 2, 3, and 4 days after addition of the cytokine. On days 2 and 3, higher doses of 100U/ml and 1000U/ml IFNγ appeared to be causing an increase in protein synthesis, with the effect being most marked at the highest

dose. Because of the correction factors which had to be included, no statistical analysis was possible. However, these doses of IFNγ caused the uptake of ^{3}H leucine to almost double compared with controls. By day 4, all concentrations of IFNγ stimulated protein synthesis to levels at least double those of controls, although there was no clear dose response.

Fig. 2 shows the effect of TNFα on protein synthesis. On all days, none of the concentrations of TNFα had any effect on protein synthesis by the RIE-1 cells compared with controls.

Neither TGFβ (Fig. 3), nor IL1 (Fig. 4) had any effect on protein synthesis at any concentration or at any of the time points assessed.

Effects of IFNγ and TNFα on the Expression of MHC Molecules on the RIE-1 Cells

Enteropathies such as coeliac disease are associated with *de novo* expression of MHC class II molecules by crypt enterocytes (Arnaud-Battandier et al., 1986) and it is thought that this is an effect of IFNγ. As I found that IFNγ increased total protein synthesis by RIE-1 cells, I next examined the effects of IFNγ on the expression of MHC molecules by RIE-1 cells. In addition, I investigated the effects of TNFα on MHC expression, as TNFα is known to synergise with the action of IFNγ in other systems.

RIE-1 cells were cultured until confluence and then incubated with either 1000U/ml of IFNγ and/or 1000U/ml of TNFα, or in medium alone. These concentrations of cytokine were used as I had previously shown that they had consistent and marked effects on the growth of RIE-1 cells and, in the case of IFNγ, an effect on total protein synthesis. 24 hrs later, the cells were stained by immunofluorescence

for the expression of MHC class I and II molecules and analysed by flow cytometry.

Control cells stained for MHC class I molecules showed an increase in the fluorescent intensity compared with unstained cells, indicating a low level of constitutive expression of MHC class I molecules on RIE-1 cells (Fig. 5a). Incubation of the cells with IFNγ alone caused a marked increase in the expression of class I molecules, with virtually all RIE-1 cells now expressing very high levels (Fig. 5b). Addition of TNFα alone caused a slight increase in expression of MHC class I molecules by most cells compared with controls, but this was much less than the effects of IFNγ (Fig. 5c). When RIE-1 cells were incubated with both IFNγ and TNFα, a greater effect was seen than that obtained with either cytokine alone, with all cells expressing high levels of MHC class I molecules and the peak of the population being off the scale of the flow cytometer (Fig. 5d). Thus, IFNγ and TNFα both increase the constitutive expression of class I MHC molecules by RIE-1 cells.

Having measured expression of class I MHC molecules, I then proceeded to examine class II MHC expression by the RIE-1 cells. Untreated RIE-1 cells stained with antibody against class II molecules showed no increased staining compared with control cells stained with secondary antibody only, indicating that they do not express MHC class II molecules constitutively (Fig. 6a). IFNγ alone caused about a quarter of the cells to express moderately high levels of class II molecules (Fig. 6b), but TNFα alone had no effect on the expression of MHC class II molecules (Fig. 6c). Addition of both IFNγ and TNFα caused a synergistic increase in the expression of class II molecules, with most cells now expressing class II molecules (Fig. 6d). Although

the level of expression by the cells was rather variable and generally less than that of class I MHC molecules, a substantial number of the cells treated with both cytokines had high levels of class II molecules. Thus, IFNγ can induce the *de novo* expression of MHC class II molecules and this effect is enhanced by TNFα, which itself has no effect.

The Effects of TGFβ and IFNγ on the Ultrastructural Morphology of RIE-1 Cells

The effects of IFNγ on protein expression and MHC expression would be consistent with an ability of this cytokine to induce differentiation of immature RIE-1 cells. I therefore examined IFNγ treated cells for ultrastructural evidence of differentiation, including polarisation, appearance of microvilli and increased complexity of cytoplasmic organelles. In these experiments, I also investigated the effects of TGFβ, as this cytokine causes differentiation in a number of cell types *in vitro* and its inhibitory effects on the growth of intestinal epithelial cells are thought to be linked to differentiation.

RIE-1 cells were cultured in the presence of100U/ml IFNγ or, 10ng/ml TGFβ, or medium alone and then processed for EM 4 days later, by which point all the cultures were confluent. These concentrations of cytokine were used, as I had previously shown that they had consistent and marked effects on proliferation of RIE-1 cells. As described in chapter 1, control RIE-1 cells exhibited a flattened morphology with desmosomes and tight junction structures between adjacent cells, but with very few microvillus-like projections and a paucity of cytoplasmic organelles (Fig. 7). In the current experiments, treatment with either TGFβ (Fig. 8) or IFNγ (Fig. 9) had no effect on the

morphology of RIE-1 cells. Cytokine treated cells retained a flattened morphology with desmosomes and tight junctions and there was no obvious change in the morphology or frequency of the microvillus-like projections. Thus, neither TGFβ nor IFNγ appear to induce differentiation of RIE-1 cells.

Formation of Impermeable Monolayers by Epithelial Cells

A critical feature of the intact intestinal epithelium *in vivo* is its impermeability to macromolecules, largely due to the formation of tight junctions between adjacent cells. This property is reproduced by certain differentiated enterocyte cell lines *in vitro* and tight junction function can be measured by calculating the resistance to a voltage across confluent cell layers. As it has been reported that the tight junction function of differentiated epithelial cells can be influenced by cytokines (Adams et al., 1993, Holmgren et al., 1989), I investigated whether RIE-1 cells would form a monolayer of cells with functional tight junctions and if this could be affected by cytokines.

RIE-1 cells were grown in transwell chambers and the resistance to a voltage applied across the cells was measured over a 2 week period. As a positive control, Caco-2 cells were also used in this experiment, as it is known that these cells form functional tight junctions.

Initially, Caco-2 cells had no resistance above background, but by day 10, when the cells had been confluent for several days, their resistance was much greater than background, indicating the presence of intact tight junctions (Table 1). In contrast, RIE-1 cells never showed any resistance above background, even when grown to one week after confluence. Thus, although RIE-1 cells have structures resembling tight junctions on transmission EM photographs, they do

not form functional tight junctions as measured by electrical resistance. This result emphasises the immature nature of the RIE-1 cell line and precluded further studies using cytokines.

Conclusions

The experiments in this chapter were designed to investigate whether immune mediators could influence the differentiation of dividing epithelial cells. As differentiation necessitates an increase in protein synthesis by the cells, I first investigated the effects of cytokines on the total protein synthesis of RIE-1 cells, using those cytokines which had influenced the growth of RIE-1 cells. TGFβ, IL1, and TNFα had no effect on total protein synthesis, despite the fact that they influenced the growth and proliferation of RIE-1 cells. In contrast, all concentrations of IFNγ caused an increase in protein synthesis. Together with the fact that the cytostatic effect of IFNγ meant that there were fewer RIE-1 cells in IFNγ treated cultures than in controls, these results indicated that each cell was synthesising a greater amount of protein. Thus, IFNγ may influence both growth and differentiation of enterocytes *in vivo* and could account for the increased rate of differentiation by epithelial cells seen in enteropathy.

The MHC molecules are of particular interest in enteropathy as *de novo* expression of class II MHC proteins occurs on crypt epithelial cells under these conditions. The effects of IFNγ which I observed on total protein synthesis were accompanied by an increased expression of both class I and class II MHC molecules by RIE-1 cells. TNFα alone caused a small increase in expression of MHC class I molecules, but had no effect on class II expression. However, TNFα enhanced the

effects of IFNγ in both cases, consistent with the fact that TNFα synergises with IFNγ in other systems (Campbell et al., 1988, Weetman and Rees, 1988).

Despite the effects of IFNγ on total protein synthesis and MHC expression, no morphological evidence of differentiation was observed on ultrastructural analysis of IFNγ treated RIE-1 cells. TGFβ, another mediator thought to be important in enterocyte differentiation *in vivo*, also had no effect on RIE-1 cell diffrentiation *in vitro*, as assessed by modulation by regulatory mediators. However, it is tempting to speculate that the increased protein synthesis and expression of MHC molecules induced by IFNγ might represent the initial events of differentiation. Their lack of capacity to differentiate into mature absorptive cells may be due to the fact that RIE-1 cells have the ability to divide indefinitely *in vitro*.

From these results,I concluded that although the RIE-1 cell line provides a useful model to study enterocyte growth, it could not be used on its own to study other functions such as differentiation. Therefore in the next chapter, I attempted to produce a model closer to that seen *in vivo*, in which mesenchymal cells were co-cultured with the RIE-1 cells in an attempt to provide further differentiation.

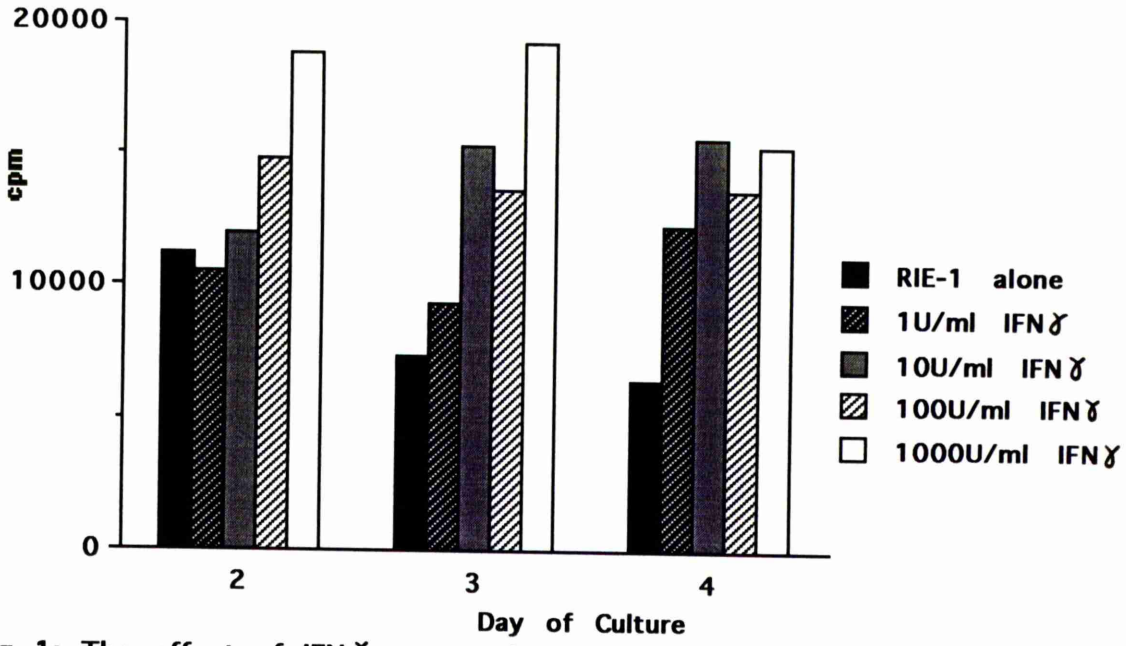

Fig 1: The effect of IFNγ on protein synthesis by confluent cultures of RIE-1 cells as measured by tritiated leucine uptake. Results shown are mean cpm of quadruplicate wells/10^6 cells and thus no statisitics have been calculated.

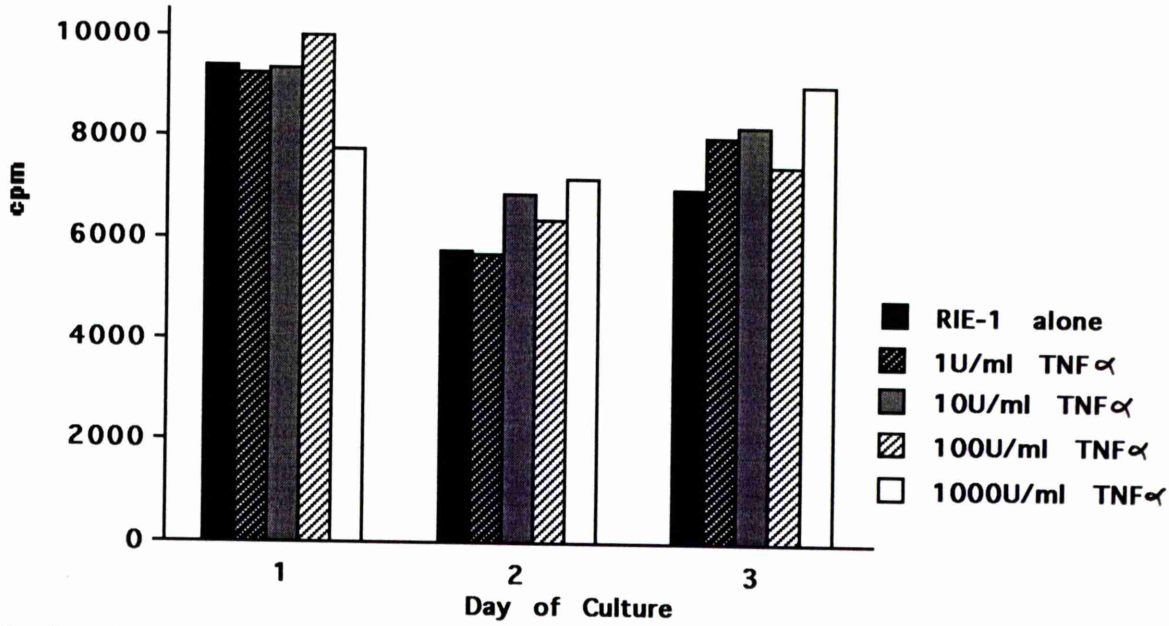

Fig 2: The effect of TNFα on protein synthesis of confluent cultures of RIE-1 cells as measured by uptake of tritiated leucine. Results shown are mean cpm of quadruplicate wells/10^5 cells and thus no statistics have been calculated.

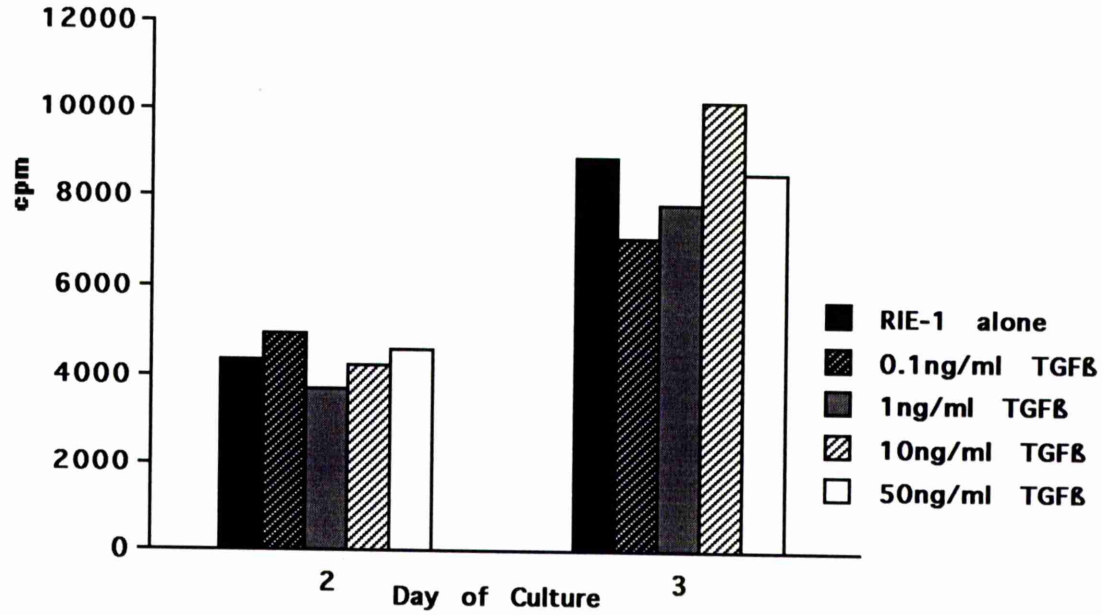

Fig 3: The effect of TGFß on protein synthesis by confluent cultures of RIE-1 cells as measured by uptake of tritiated leucine.
Results shown are mean cpm for quadruplicate wells/10^5 cells and thus no statisitics are calculated.

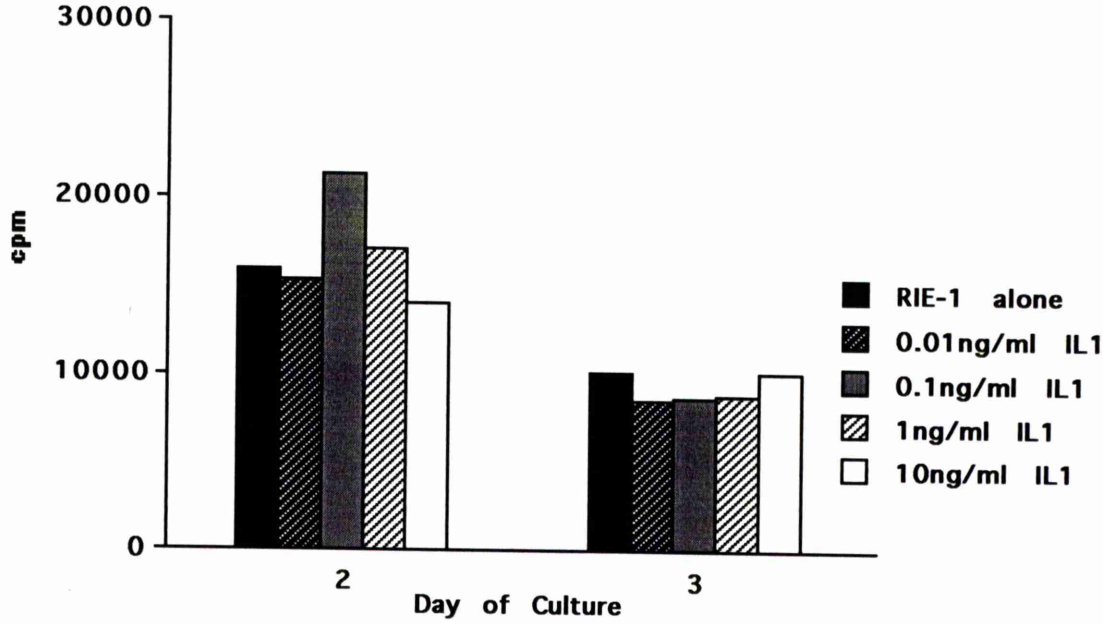

Fig 4: The effect of IL1 on protein synthesis by confluent cultures of RIE-1 cells as measured by uptake of tritiated leucine.
Results shown are mean cpm for quadruplicate wells/10^5 cells and thus no statistics are calculated.

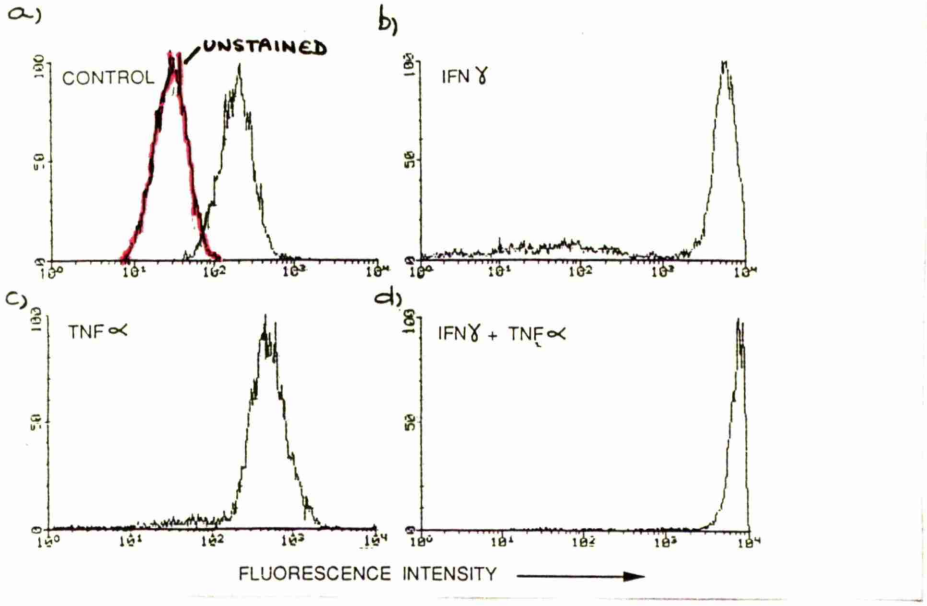

Fig: 5 FACS profiles of RIE-1 cells treated with 1000U/ml IFNγ and TNFα and stained for MHC class I using the OX18 antibody.

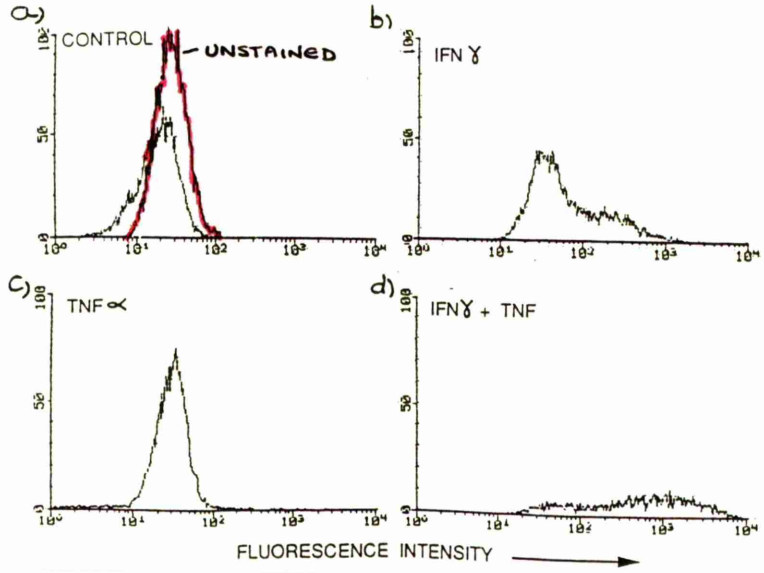

Fig: 6 FACS profile of RIE-1 cells treated with 1000U/ml IFNγ and TNFα stained for MHC class II molecules with OX6 antibody.

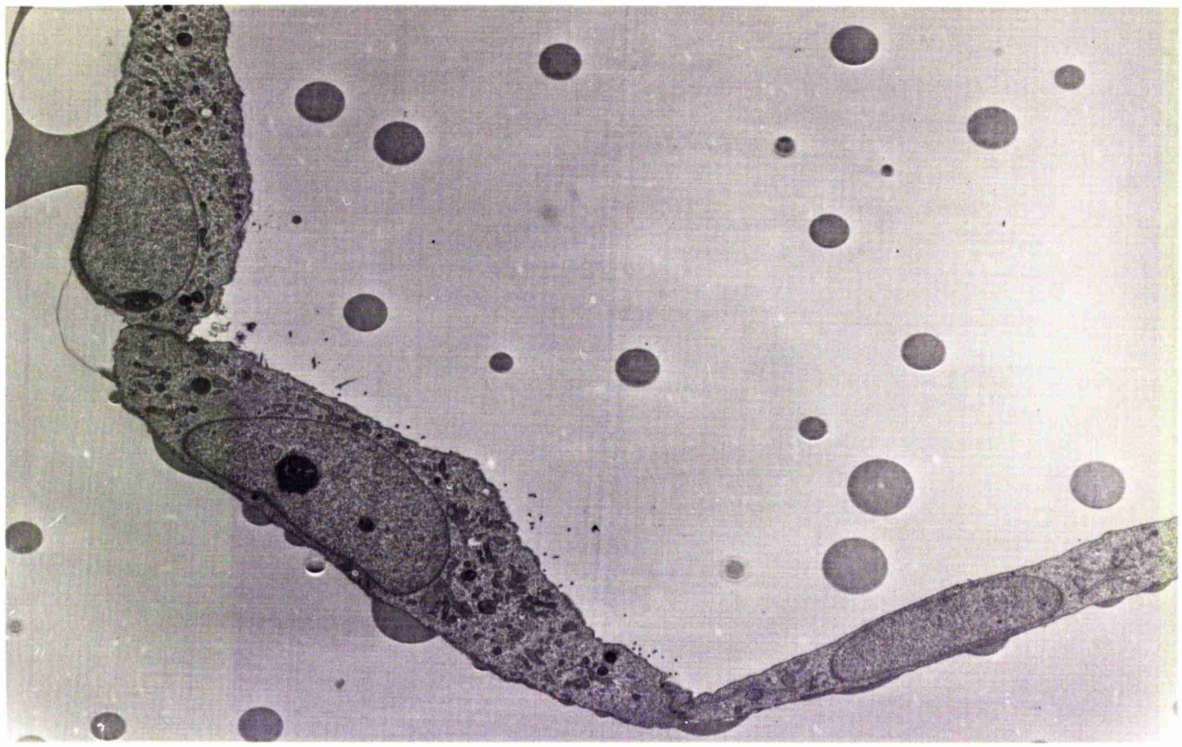

Figure 7: Electron micrograph of a transverse section through a confluent culture of control RIE-1 cells (x 3300)

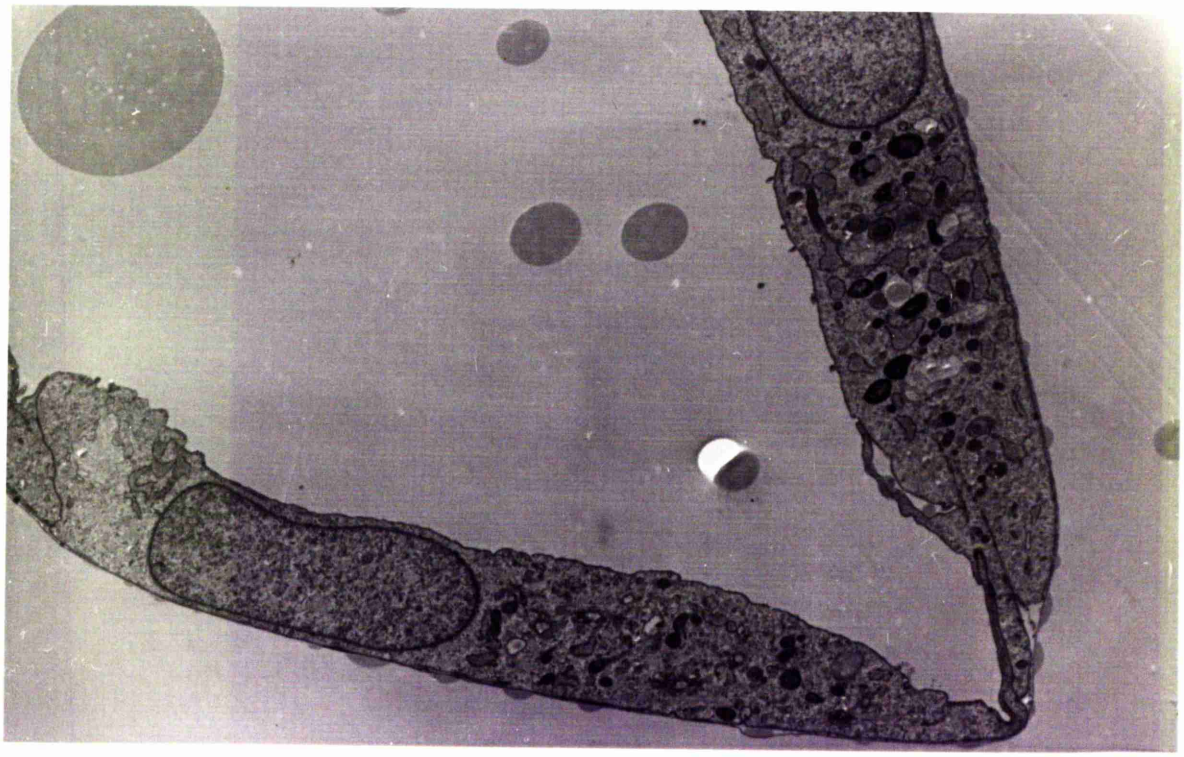

Figure 8: Electron micrograph of a transverse section through a confluent culture of RIE-1 cells treated with 10ng/ml TGFβ (x5900)

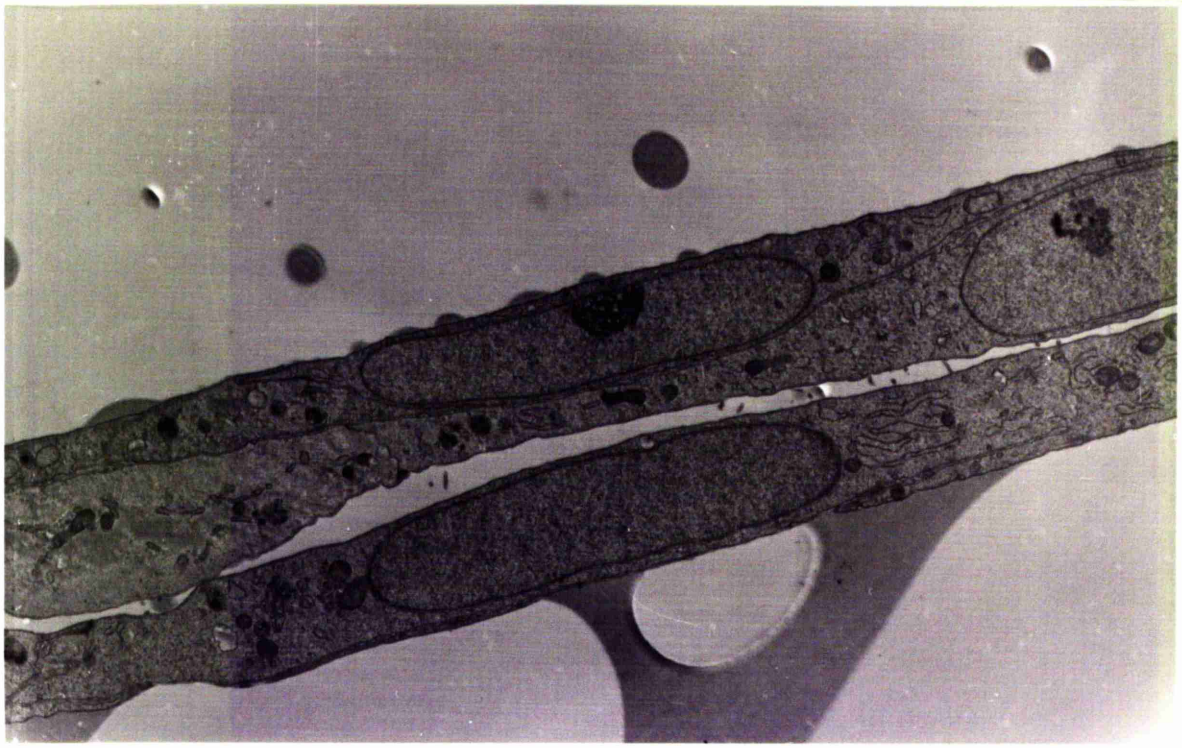

Figure 9: Electron micrograph of a transverse section through a confluent culture of RIE-1 cells treated with 100U/ml IFNγ (x5900)

CELL LINE	day 3	day 12	day 14
RIE well 1	315 ohm	300 ohm	300 ohm
RIE well 2	330 ohm	310 ohm	330 ohm
Caco-2 well 1	310 ohm	630 ohm	750 ohm
Caco-2 well 2	320 ohm	700 ohm	700 ohm

Table 1: The resistance over time of RIE and Caco-2 cell cultures grown in individual transwells as measured using a voltmeter. The resistance of the transwell itself is approximately 300 ohm.

CHAPTER 7
CO-CULTURE OF RIE-1 CELLS WITH FIBROBLASTS

Introduction

The results of the preceding chapters have shown that RIE-1 cells provide a means of studying the effects of immune cells and their products on dividing crypt epithelial cells. However, I was unable to induce differentiation in these cells when cultured alone, despite the fact that this is an important consequence of the immune response *in vivo*. It is known that growth and differentiation of epithelial cells *in vivo* requires interactions between enterocytes and mesenchymal cells. Therefore, in this chapter, I attempted to establish a co-culture system which might allow RIE-1 cells to differentiate *in vitro* and in which the effects of cytokines could be assessed. As no rat mesenchymal cells were available and it has been shown in other systems that species specificity of mesenchyme is not required in co-culture with endoderm, I used L929 murine fibroblasts as a source of mesenchymal cells. I first assessed whether the MTT and ^{3}H-TdR assays which I used in previous chapters were applicable for measuring the growth and proliferation of the RIE-1 cells in co-culture. I then investigated whether soluble fibroblast products could have any effect on RIE-1 cell growth and, finally, I looked at the differentiation of the cells in the co-cultures using transmission EM.

Results

Growth and Proliferation of RIE-1 Cells in Co-culture with Fibroblasts

RIE-1 and L929 cell co-cultures were set up by seeding L929 cells into wells and allowing them to grow to confluence. Unless otherwise stated, the L929 cells were irradiated before addition of RIE-1 cells in culture medium. Co-cultures were then harvested 2, 3, and 4 or 5 days later using an MTT or ^{3}H-TdR proliferation assay.

In the first experiments, 10^4 RIE-1 cells were used in the co-cultures. Irradiated L929 cells used in the experiment were still able to maintain a small, but relatively constant uptake of ^{3}H-TdR (Fig. 1). However, the RIE-1 cells showed much higher levels of proliferation throughout the culture period, which reached a maximum on days 4 and 5. This was abolished when RIE-1 cells were co-cultured with fibroblasts, with ^{3}H-TdR uptake values for the co-cultures being comparable with those for L929 cells alone. Measurement of MTT uptake showed that there was no significant increase in the number of irradiated L929 cells alone over the 3 day culture period, whereas the number of RIE-1 cells increased significantly when cultured alone (Fig. 2). MTT uptake by the co-cultures was never significantly different from that of the L929 cells alone, supporting the view that fibroblasts were preventing the RIE-1 cells from growing.

I had anticipated that the RIE-1 cells would grow on top of the fibroblast monolayer. However, on viewing the cells by light microscopy, it was difficult to see any growing RIE-1 cells. Indeed, the RIE-1 cells did not even appear to flatten and attach to the L929 cells as they did on a plastic substratum. To explore whether the failure to measure growth was because too few RIE-1 cells were attaching, in the next experiment I used $3x10^4$ RIE-1 cells. Once again, L929 cells had a residual level of proliferation as measured by ^{3}H-TdR uptake and RIE-1 cells alone proliferated well (Fig. 3). In addition, ^{3}H-TdR uptake by the co-cultures was comparable to the uptake by L929 cells alone, suggesting that the RIE-1 cells were not proliferating. On days 2 and 3 of culture, the uptake of MTT by the co-cultures was also not significantly different to that of L929 cells alone (Fig. 4). However, on day 4, the co-cultures showed a

significantly higher MTT uptake than L929 cells alone, indicating the presence of viable RIE-1 cells. However, this was lower than the addition of individual values. Light microscopic examination confirmed these findings by showing that some RIE-1 cells were pushing under the fibroblast monolayer and growing on the plastic substratum, although it was difficult to distinguish conclusively between the RIE-1 and L929 cells.

To explore whether the fibroblasts were producing a soluble mediator that was cytotoxic or inhibitory to the growth of the RIE-1 cells, I examined the effects of fibroblast conditioned medium on the growth of RIE-1 cells. The growth of RIE-1 cells cultured in 50% L929 conditioned medium was not significantly different from the growth of cells cultured alone (Fig. 5), indicating that the L929 cells were not producing cytotoxic soluble mediators.

As the RIE-1 cells did not grow on top of a monolayer of L929 cells, it appears that this co-culture system would not be a useful model for studying the effects of cytokines on RIE-1 cells associated with mesenchymal cells.

Transmission EM Examination of Cultures of RIE-1 Cells and L929 Cells

I next examined the co-cultures by transmission electron microscopy, not only to obtain a more accurate picture of the fate of RIE-1 cells in the co-culture system, but also to investigate whether any of the surviving RIE-1 cells showed evidence of differentiation.

The cultures of RIE-1 and irradiated L929 cells were studied on days 2, 3, 4 and 7 days of culture. At all times, RIE-1 cells cultured alone showed their typical non-polarised flattened morphology with a smooth upper surface and a few rudimentary microvilli, as described

in chapter 1 (Fig. 6). The basal surface of the cells was closely adherent to the plastic surface.

After 2 days of co-culture with L929 cells, the RIE-1 cells had a very different appearance. The fibroblasts could be identified as a very flat layer of cells with few distinguishing features and, at this time, a number of the RIE-1 cells had detached from the fibroblast layer (Fig. 7). These cells were circular in shape and had a more irregular surface than that of RIE-1 cells cultured alone. Although some of the cells were sitting on the fibroblast layer, there was no evidence of attachment to the fibroblasts. There were also no tight junctions or desmosomes between the epithelial cells, although the membranes remained closely associated (Fig. 8). There was no clear evidence of differentiation of the RIE-1 cells, which remained unpolarised, with only a few rudimentary projections scattered randomly around the cell membrane. However, compared with control cells, the co-cultured RIE-1 cells did appear to have an increased complexity of cytoplasmic organelles, perhaps indicating some level of differentiation.

A similar pattern was seen on day 3 in the co-cultures, although by this time the RIE-1 cells appeared to have pushed aside the fibroblasts to gain access to the plastic surface and it was increasingly difficult to distinguish between the cell populations (Fig. 9). Some of these cells were obviously epithelial in origin as they had desmosomes between cells and were similar in morphology to control RIE-1 cells. Many other epithelial cells remained round in shape and some had now become almost columnar in shape, with the nucleus situated towards the bottom of the cell. This might be early evidence of polarisation of the cells (Fig 10). These rounded up cells also now formed a more complex two-layered structure, with a lower layer

remaining in close contact with the flattened basal cell layer (which may have been epithelial and/or fibroblastic in origin) . The detached epithelial cells were closely associated with each other, but there was still no evidence of tight junctions or desmosomes. They also contained a high density of cytoplasmic organelles, including mitochondria and vesicles (Fig 11).

The appearances of co-cultures on day 4 were generally similar to those on day 3 (Fig 12), except that in some areas there was rather more evidence of disordered masses of epithelial cells of mainly rounded appearance (Fig 13, 14). Some of these cells were connected to one or more adjacent cells, while others were completely detached. By this time, no fibroblast cells were visible and it possible that many of them had died.

Day 7 co-cultures showed a thick layer of tightly packed epithelial cells, with no fibroblasts visible (Fig. 15). The RIE-1 cells were very closely associated with one another, but again, there was no evidence of tight junctions or desmosomes. A number of the cells were columnar in shape, with the nucleus positioned in the bottom of the cell, while the upper half of the cytoplasm contained a high density of cytoplasmic organelles. Again these features are consistent with a degree of polarisation (Fig. 16). Many of the cells had a large increase in the number of vesicles in the cytoplasm (Fig. 17). It should be noted that in all co-cultures there was no increase in microvillus like structures on all days. Similar results were obtained irrespective of whether RIE-1 cells were co-cultured with irradiated or unirradiated fibroblasts.

Conclusions

The aim of this chapter was to co-culture RIE-1 cells on a fibroblast monolayer, which might resemble more closely small intestinal epithelium *in vivo*. It was predicted that the RIE-1 cells would grow on the L929 monolayer and themselves form a confluent monolayer on the surface.

However, RIE-1 cells did not proliferate when seeded onto a monolayer of L929 cells, when measured by uptake of either ^{3}H-TdR or MTT. Similar results were obtained when a greater number of RIE-1 cells were used and thus the L929 cells appeared to prevent the growth of RIE-1 cells. The inhibitory effect of the L929 cells was not due to the production of soluble factors and may therefore reflect some property of the fibroblasts which prevented attachment of the RIE-1 cells and hence loss of growth.

Ultrastructural examination showed that co-culture with L929 fibroblasts altered the morphology and structure of the RIE-1 cells. They appeared unable to attach to the fibroblast layer and either sat on top of the monolayer, or pushed through it to attach to the plastic beneath. This confirmed that RIE-1 cells could not grow as a monolayer on the surface of the L929 cells. At later times of culture, it was also not possible to identify any fibroblasts and it is likely that many had died due to the activity of those RIE-1 cells pushing through to the plastic. This suggests that some of the MTT and thymidine uptake originally attributed to the fibroblasts in the co-cultures was actually due to RIE-1 cells and that there were growing epithelial cells in the co-culture. However, their numbers must have been much lower than when RIE-1 cells were cultured alone.

RIE-1 cells in co-culture did not grow in a monolayer as did those cultured alone, but formed a more complex structure which was

several cells thick. The cells became rounded up, and a small proportion acquired a columnar appearance with the nucleus located in the bottom half of the cell and a high density of cytoplasmic organelles in the cytoplasm in the top half of the cell. This suggested that these cells were polarising and that some degree of differentiation may have been occurring. Although it should be noted that very few cells had this appearance, most epithelial cells in co-culture had an increased number of cytoplasmic organelles such as mitochondria and vesicles. Apart from these features, there was no other evidence of epithelial cell differentiation. Indeed, unlike RIE-1 cells cultured alone, those in co-culture did not have tight junctions or desmosomes. In addition, they had very few microvillus-like structures and bore little resemblance to small intestinal epithelial cells *in vivo*.

Thus, co-culture of RIE-1 cells with L929 fibroblasts did not appear to have any significant effect on the differentiation of the epithelial cells, despite the fact that they formed a multilayered structure. These results again emphasise the immature nature of RIE-1 cells, their inability to differentiate *in vitro* and their lack of suitability to measure functions other than growth of epithelial cells *in vitro*. Therefore, the next chapter describes a primary culture system of intestinal epithelial cells for use as an *in vitro* model suitable for measuring functions such as differentiation.

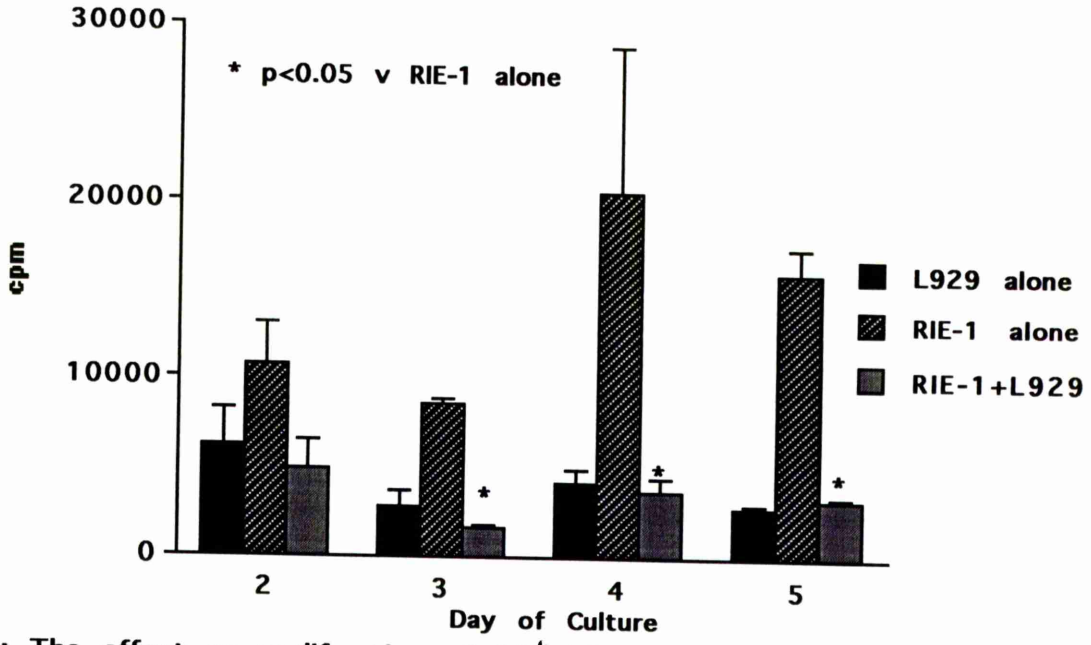

Fig 1: The effect on proliferation of 10^4 RIE-1 cells when cultured on an irradiated confluent layer of L929 cells as measured by the uptake of tritiated thymidine.
Results shown are mean cpm/well+1s.e. for quadruplicate assays.

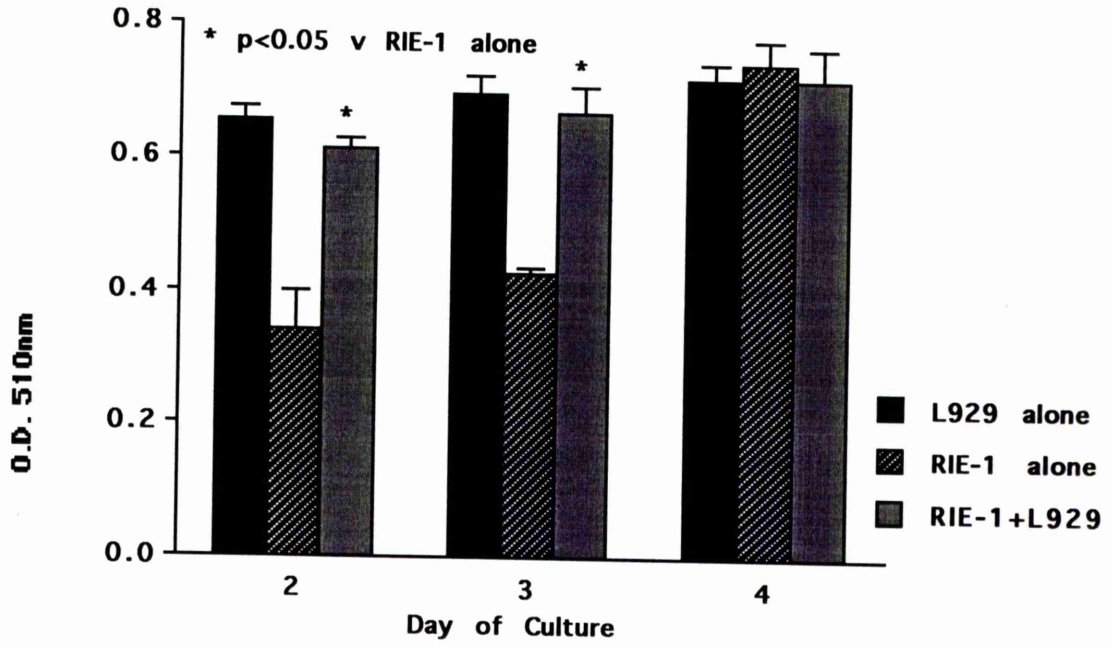

Fig 2: The effect on growth of culturing 10^4 RIE-1 cells on a confluent layer of L929 cells as measured by the MTT assay.
Results shown are mean O.D./well+1s.e. for quadruplicate assays.

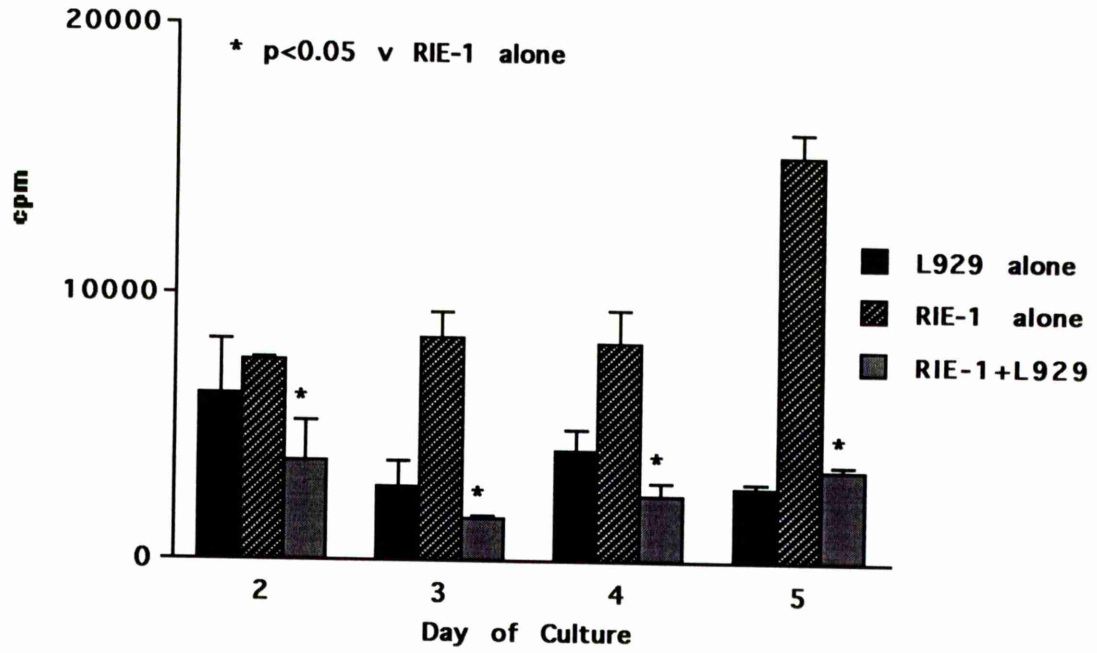

Fig 3: The effect on proliferation of culturing $3x10^4$ RIE-1 cells on a confluent layer of L929 cells as measured by uptake of tritiated thymidine. Results shown are mean cpm/well+1s.e. for quadruplicate assays.

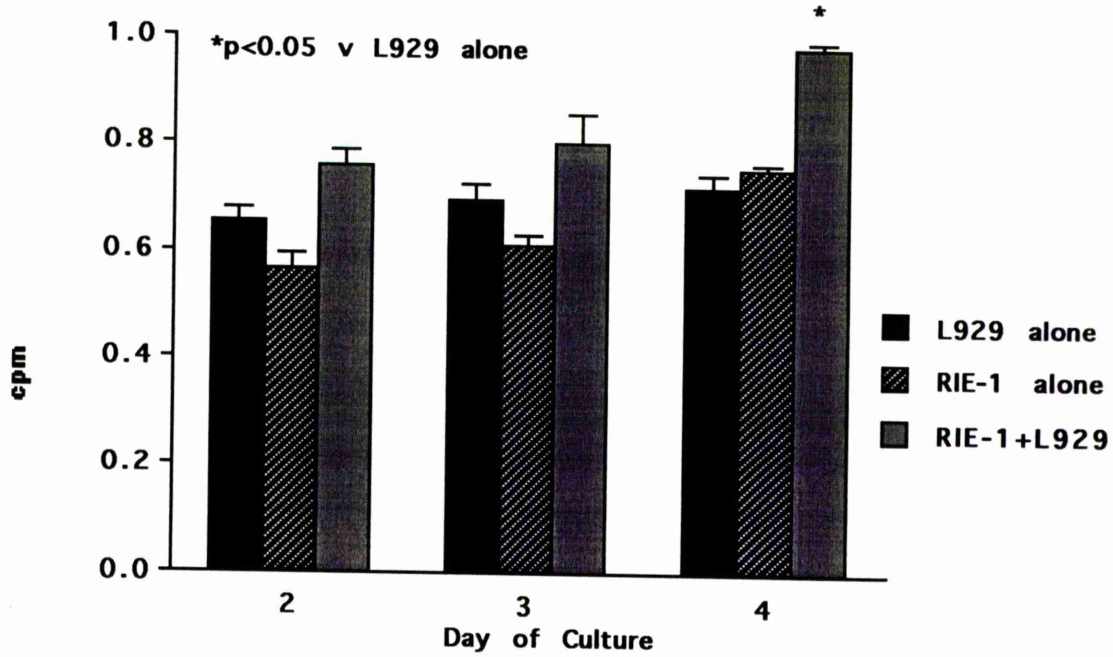

Fig 4: The effect on growth of culturing $3x10^4$ RIE-1 cells on a confluent layer of L929 cells as measured by the MTT assay.
Results shown are mean O.D./well+1s.e. for quadruplicate wells.

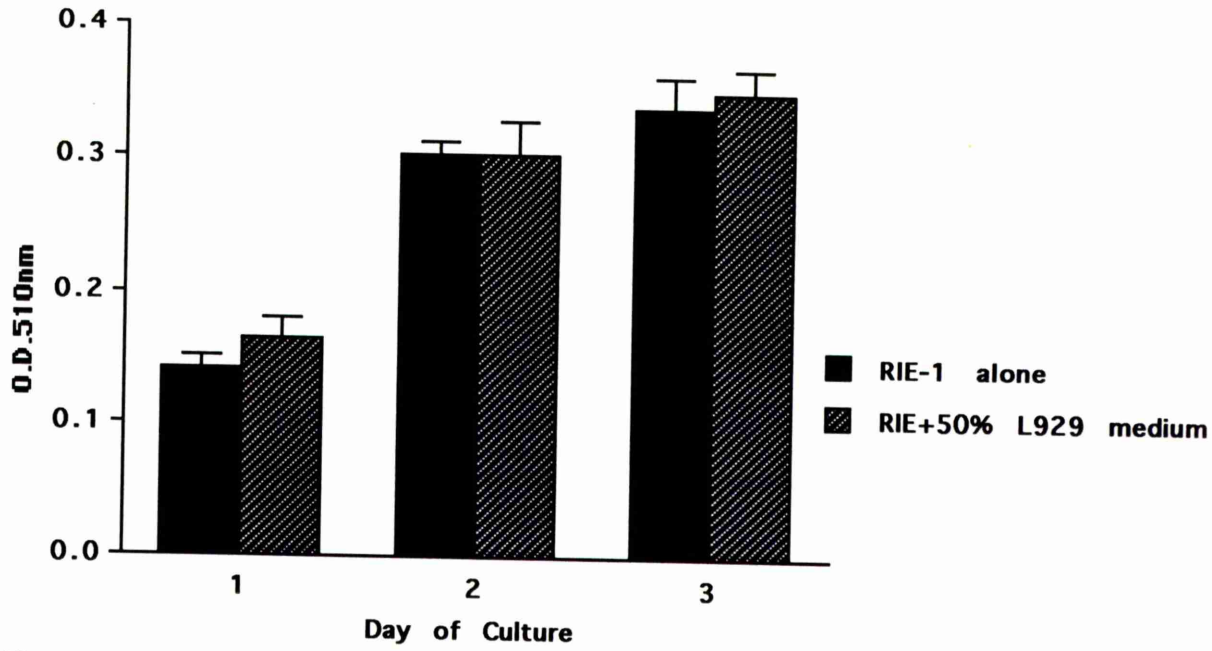

Fig 5: The effect of 50% L929 conditioned medium on the growth of RIE-1 cells as measured by the MTT assay.
Results shown are mean O.D./well+1s.e. for quadruplicate assays.

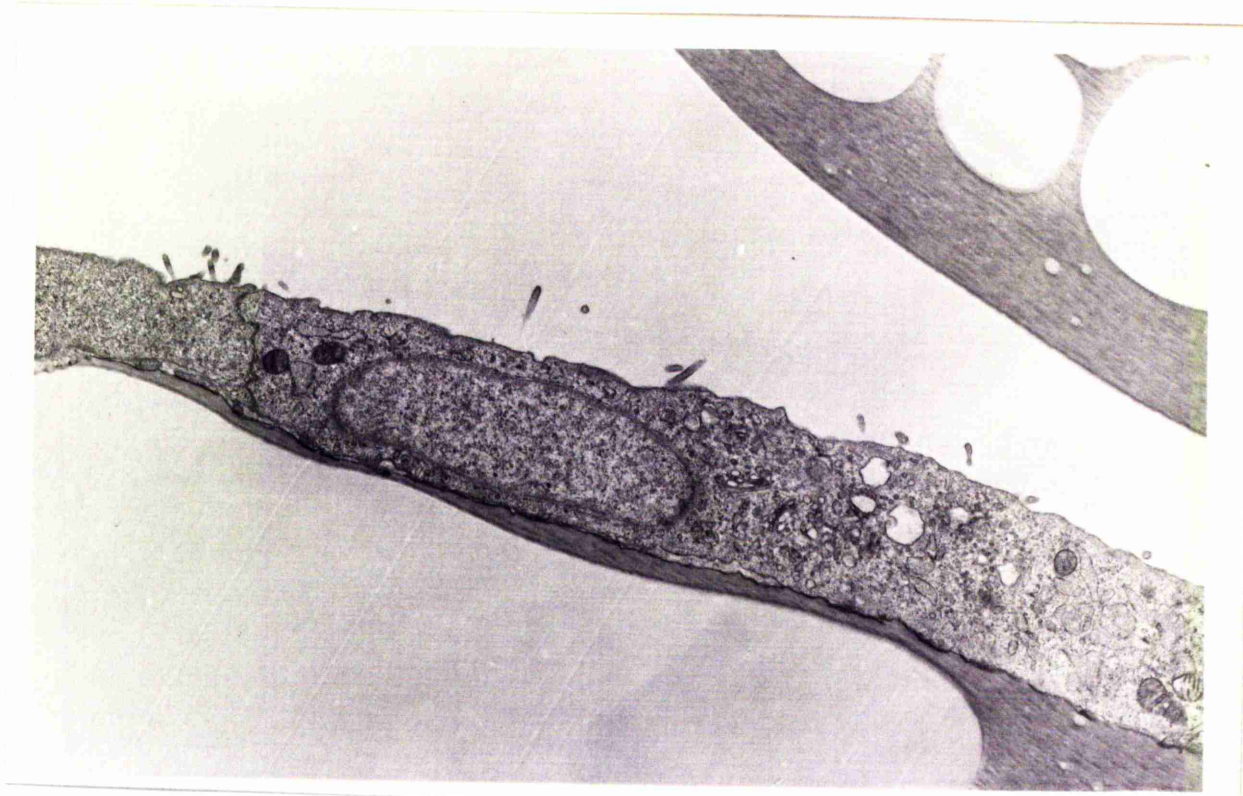

Fig 6: Electron micrograph of a transverse section through a 7 day confluent culture of RIE-1 cells (x10400)

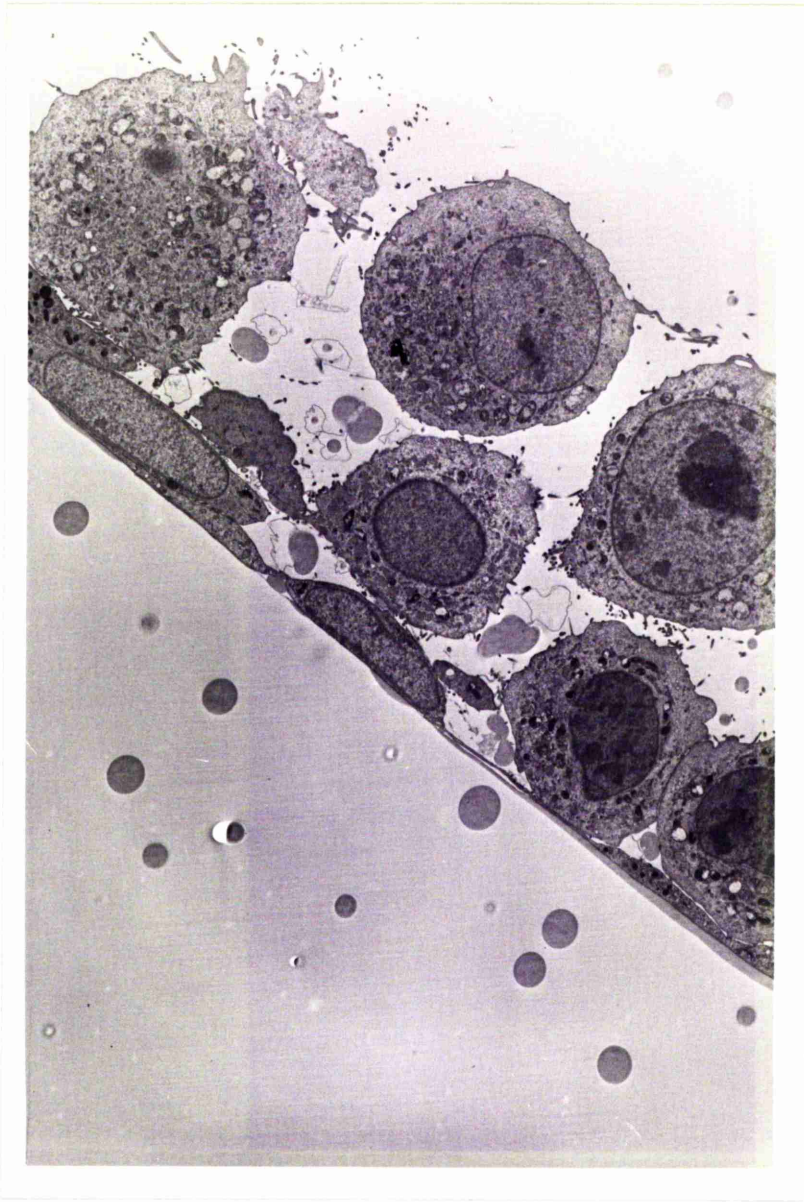

Fig 7: Electron micrograph of a transverse section through a 2 day co-culture of RIE-1 cells and L929 fibroblasts (x2400)

Fig 8: Electron micrograph of a transverse section through a 2 day co-culture of RIE-1 cells and L929 fibroblasts (x10400).

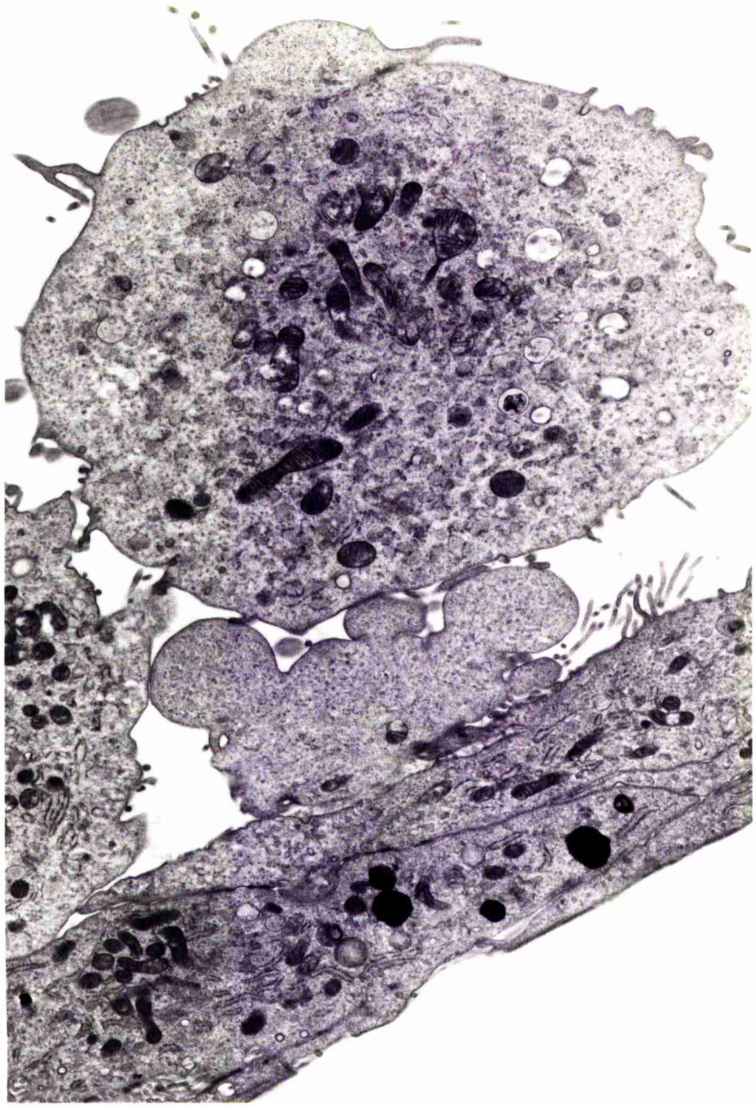

Fig 9: Electron micrograph of a transverse section through a 3 day co-culture of RIE-1 cells and L929 fibroblasts (x7800).

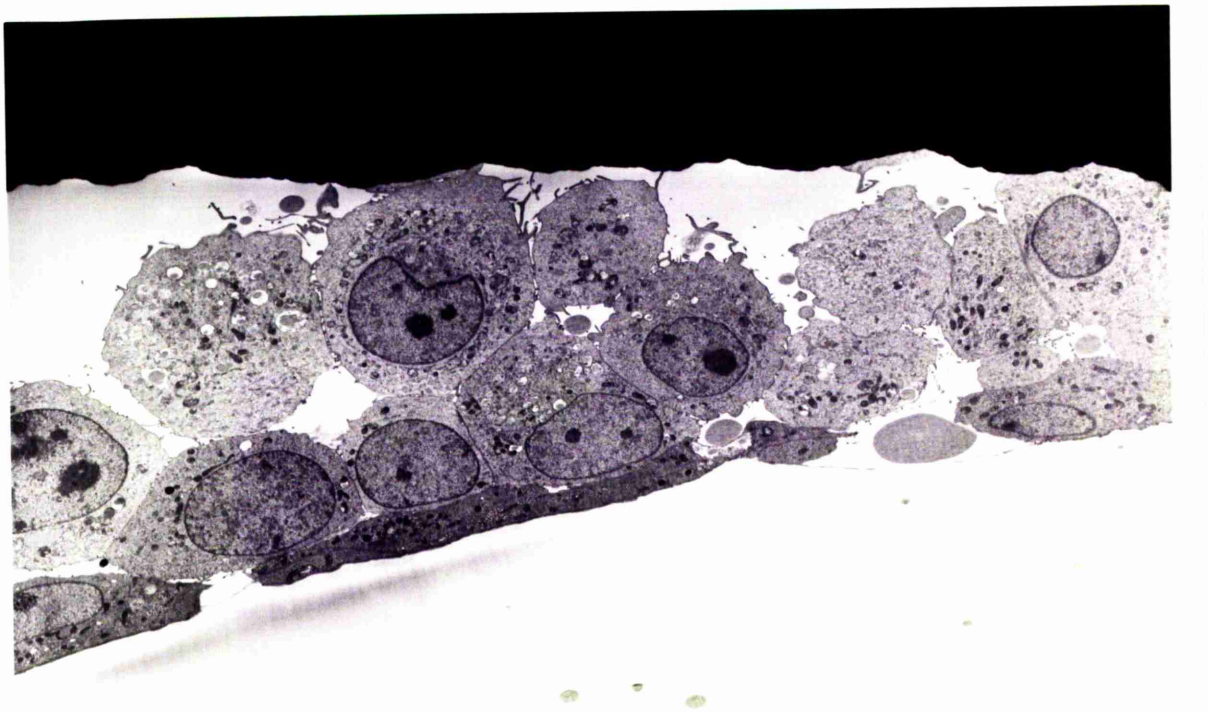

Fig 10: Electron micrograph of a transverse section through a 3 day co-culture of RIE-1 cells and L929 fibroblasts (x2400).

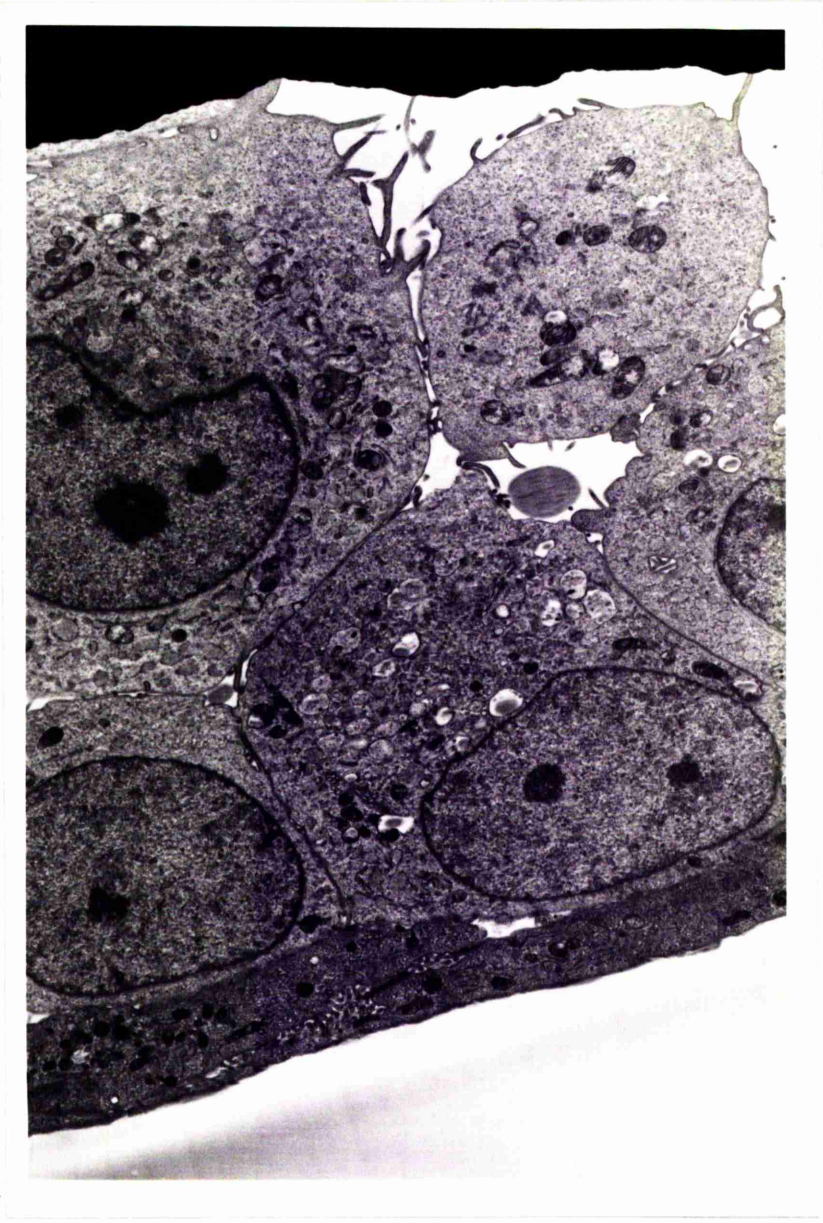

Fig 11: Electron micrograph of a transverse section through a 3 day co-culture of RIE-1 cells and L929 fibroblasts (x5900).

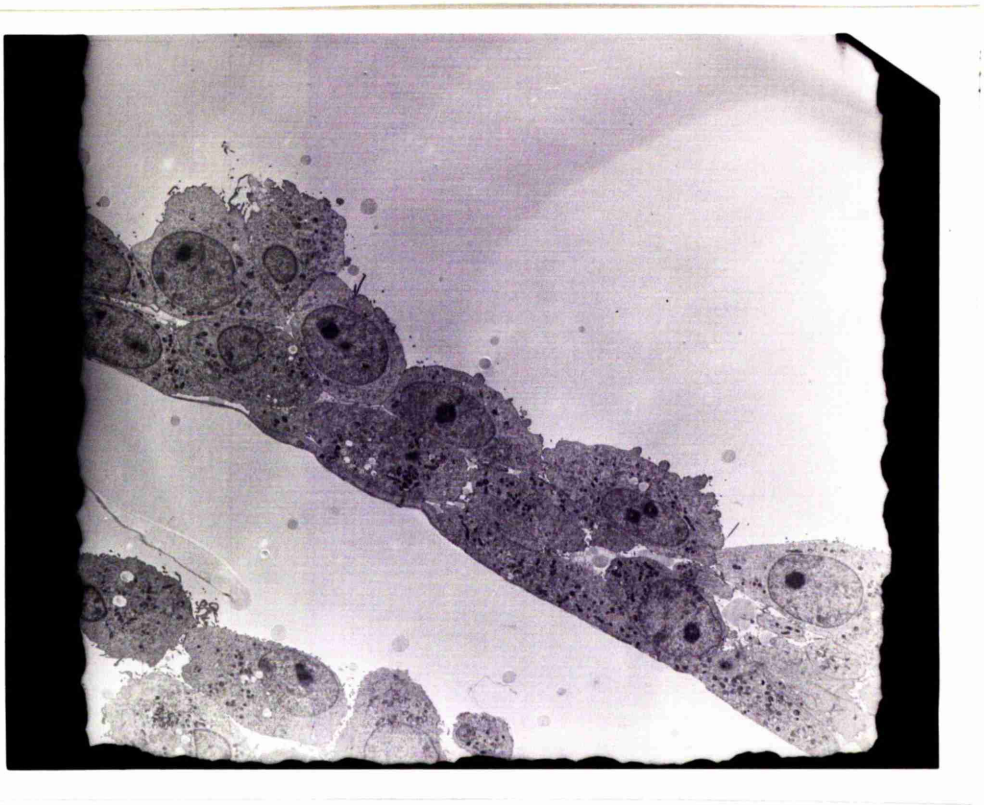

Fig 12: Electron micrograph of a transverse section through a 4 day co-culture of RIE-1 cells and L929 fibroblasts (x1400).

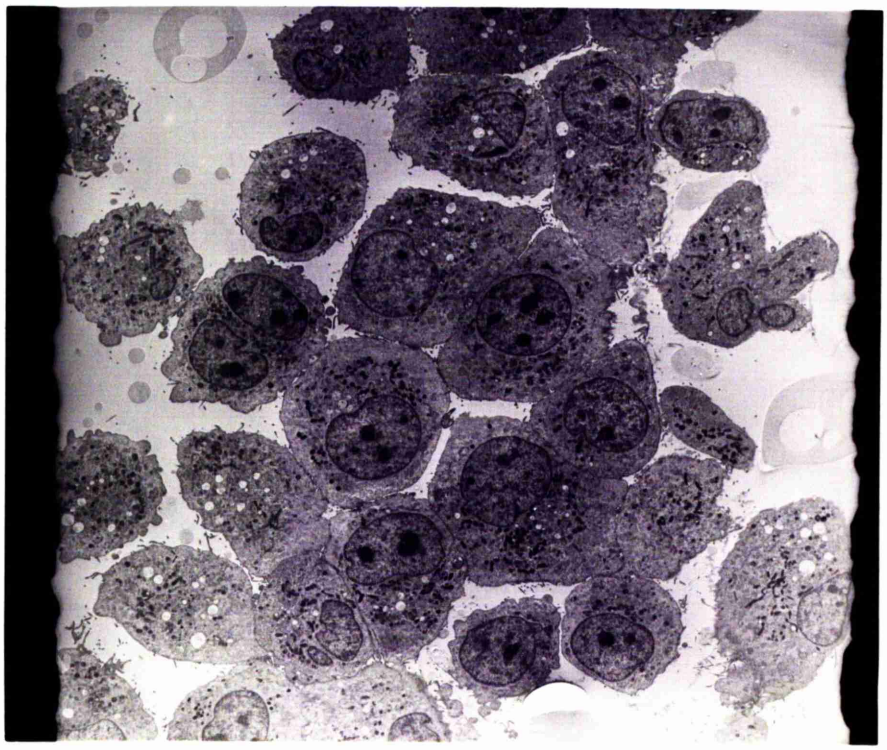

Fig 13: Electron micrograph of a transverse section through a 4 day co-culture of RIE-1 cells and L929 fibroblasts (x1400).

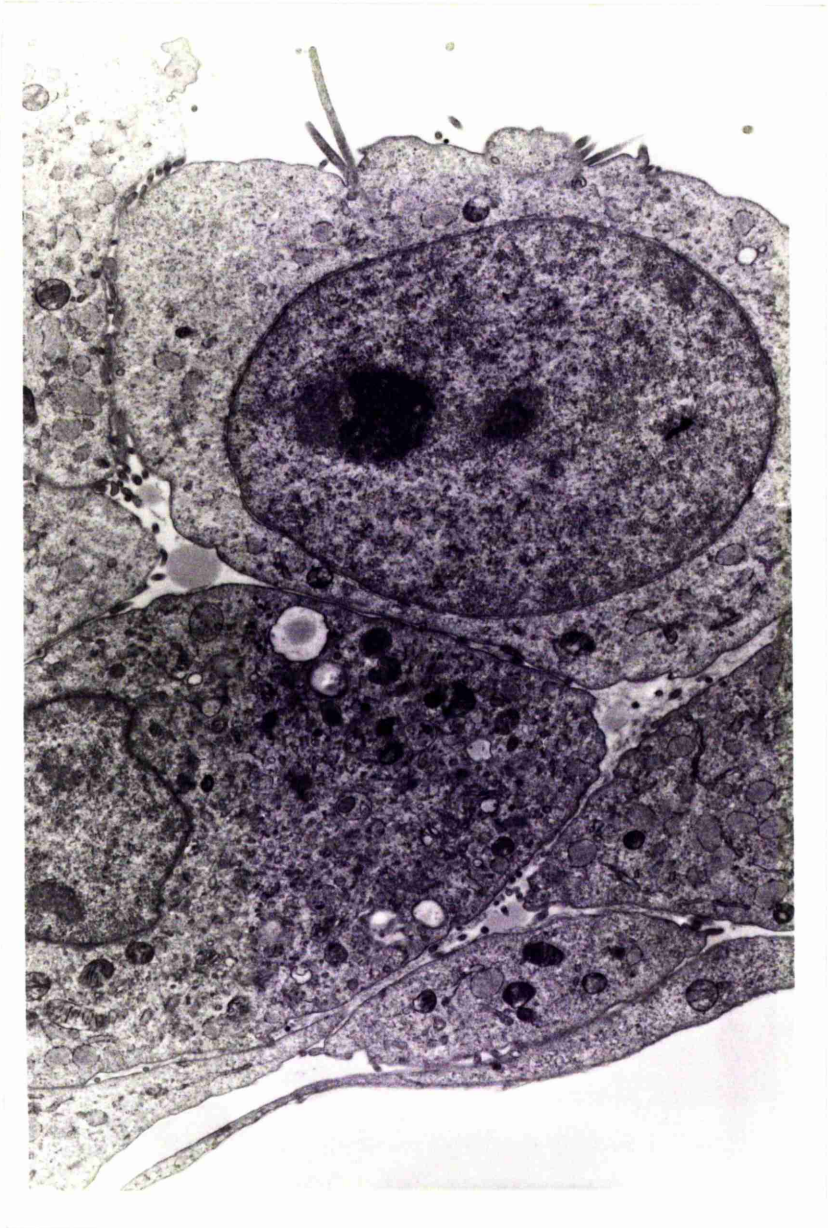

Fig 14: Electron micrograph of a transverse section through a 4 day co-culture of RIE-1 cells and L929 fibroblasts (x10400).

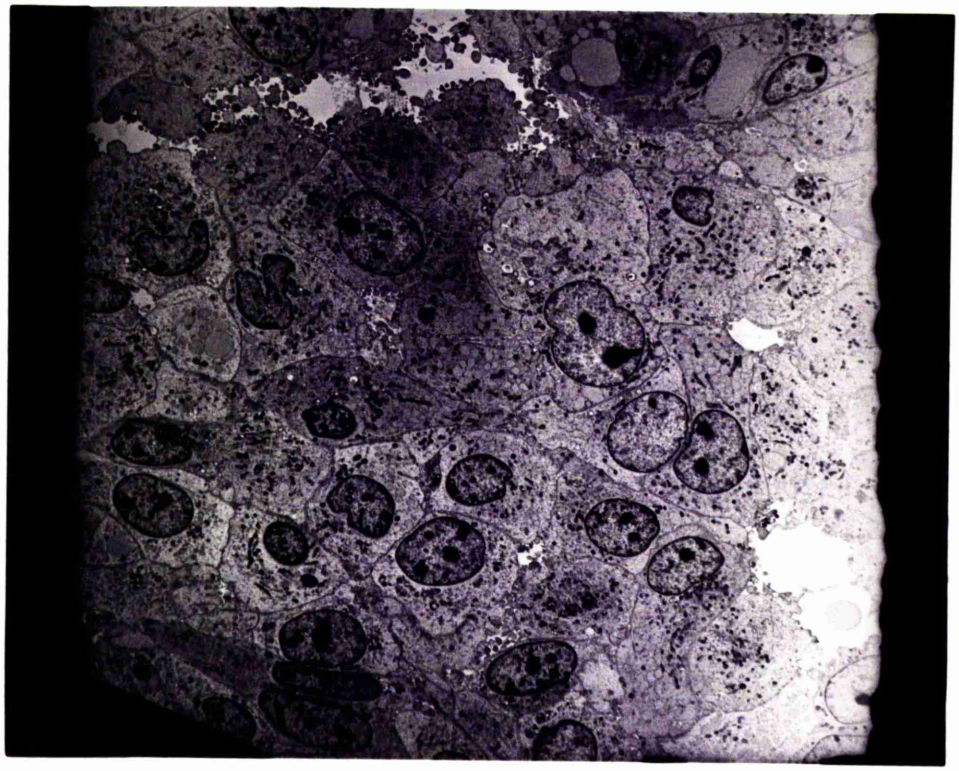

Fig 15: Electron micrograph of a transverse section through a 7 day co-culture of RIE-1 cells and L929 fibroblasts (x1400).

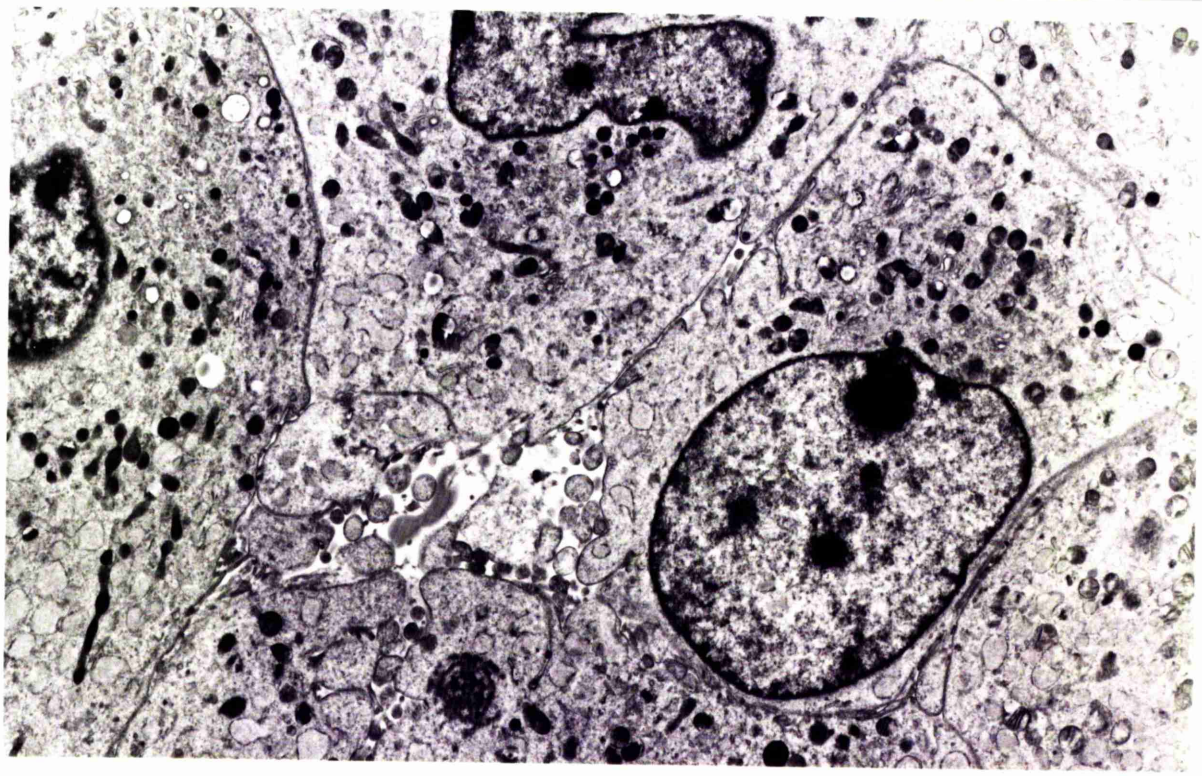

Fig 16: Electron micrograph of a transverse section through a 7 day co-culture of RIE-1 cells and L929 fibroblasts (x5900).

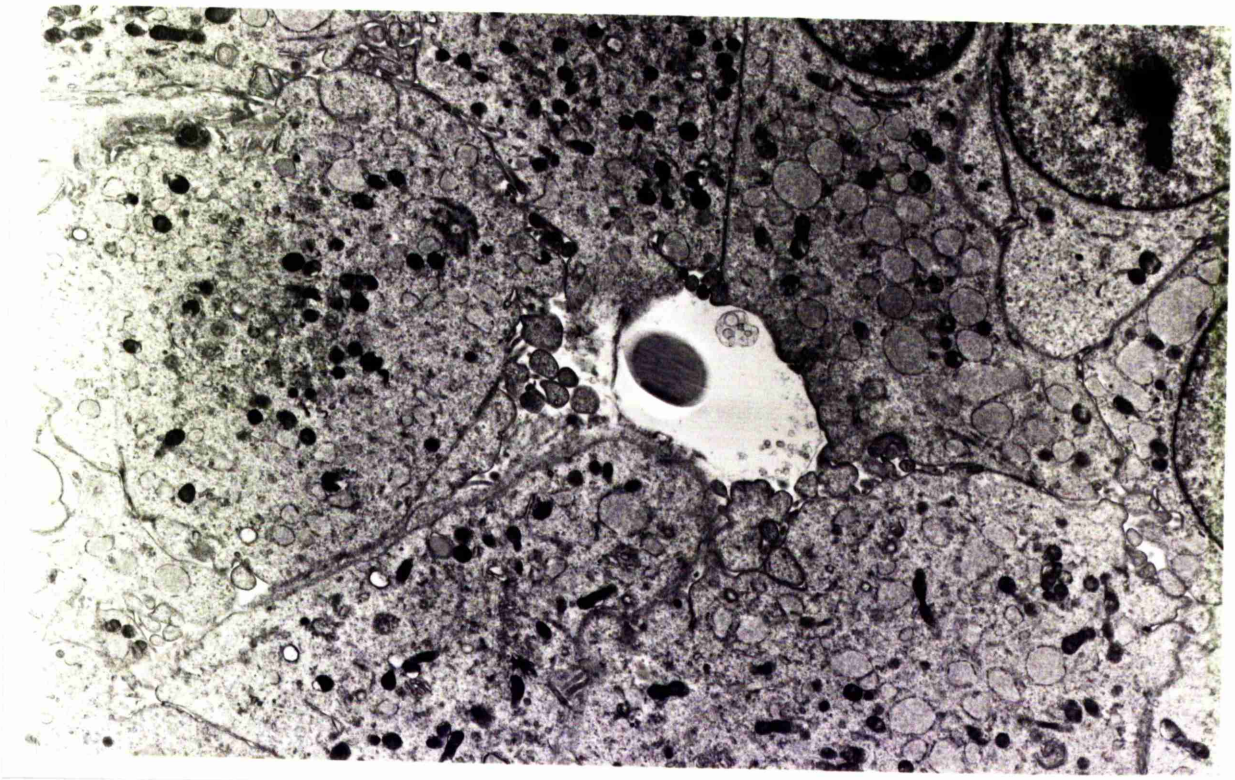

Fig 17: Electron micrograph of a transverse section through a 7 day co-culture of RIE-1 cells and L929 fibroblasts (x5900).

CHAPTER 8

ESTABLISHMENT OF PRIMARY CULTURES OF INTESTINAL EPITHELIAL CELLS

Introduction

In the previous chapters the RIE-1 cell line proved to be a useful model for studying the effects of cytokines on the growth of intestinal epithelial cells *in vitro*. However, as a cell line, the RIE-1 cells may not be entirely typical of crypt epithelial cells *in vivo* and my work has shown that the RIE-1 cell line does not appear to be suitable to study differentiation *in vitro*. Thus, in this chapter, I attempted to set up a primary culture of dividing small intestinal epithelial cells which could be used as a parallel model for studying parameters other than growth of enterocytes *in vitro*.

Initially, I assessed the growth of these primary cultures and determined their purity using light microscopy and by the expression of cytokeratins. I then used transmission EM and the expression of digestive enzymes to investigate the level of differentiation of the epithelial cells in culture and finally, I investigated the effects of immune products and a number of inflammatory cytokines on the growth of these cultures.

Results

The method I used to isolate and grow primary cultures of intestinal epithelial cells was one which had been developed by Evans and colleagues (Evans et al.,1992). When I carried out my work the method had not been established fully and the purity of the epithelial cultures was still unknown. Therefore, I first had to characterise these primary cultures in our own laboratory.

Growth and Morphology of the Primary Cultures

Epithelial cells derived from fragments of rat intestinal tissue were cultured for up to 9 days. A crystal violet colorimetric assay was used to assess the growth of the cultures and light microscopy was used to identify the cell types present.

The number of cells in the cultures increased over the culture period and by day 9 the wells were almost confluent (Fig. 1). On day 1 of culture, the cells in the tissue fragments had attached to the base of the well and were beginning to spread to form discrete islands of cells (Fig. 2). At this point, the islands consisted of tightly packed polygonal cells, suggesting that they were of epithelial origin and, in many cases, the centre of the island remained unattached to the plastic. By day 6, these islands had increased in size, and consisted of tightly packed sheets of cells which remained epithelial in appearance (Fig. 3). However, by now, there were also populations of cells which were more elongated and appeared fibroblastic (Fig. 4). By day 9, the individual islands had joined up and the cultures had almost reached confluence. Although, large areas of epithelioid cells were still present at this time (Fig. 5), large areas of the wells now contained non-epithelial appearing cells (Fig. 6). These were found particularly round the edges of the wells.

EM of Primary Cultures

As it was difficult to identify conclusively the cell types in the primary cultures using light microscopy, I used transmission EM to try and identify intestinal epithelial cells more accurately and also to assesss their level of differentiation.

On day 1, epithelial cells were clearly present, with obvious microvilli which had distinct microfilaments. However, these

microvilli were very rudimentary and did not form the dense brush border seen *in vivo* (Figs. 7 and 8). The epithelial cells also had distinct tight junctions. Some of these cells were almost columnar in shape but many were flattened and in shape resembled the RIE-1 cells. There was also a number of unidentifiable cells which may have been mesenchymal in origin or undifferentiated epithelial cells. These cells were flattened and non-polarised. Together these cells formed structures several layers thick.

On day 2, flattened layers of cells of 2 or more cells thickness were still present (Figs. 9, 10). Circular, columnar and flattened epithelial cells with distinct microvilli were found and there were also cells of unknown origin.

Day 3 showed a single layer of cells, which had well formed microvilli (Fig. 11). Higher magnification of another area of the culture showed an epithelial cell with a brush border of microvilli, which was similar in structure and density to that seen *in vivo* (Fig. 12). Microfilaments extended from the microvilli into the cytoplasm of the cell. Desmosomes were also apparent. By day 4 the cultures showed a marked change in morphology. Fig. 13 shows a section through a three cell thick layer in which most of the cells were quite flat with no obvious tight junctions or desmosomes and no microvilli and thus these cells did not appear to be epithelial cells. However, a few flattened cells with distinct tight junctions and desmosomes and sparse ill formed microvilli remained (Fig. 14). Unfortunately, due to limited access to the EM facility, I was unable to look at cultures after day 4. It is important to note that these photographs were taken from a number of sections through a culture well. Whether these sections were representative of the well as a whole was not known as

light microscopy showed that the well was not homogeneous and thus interpretation of the above results was to some extent speculative.

Expression of Cytokeratins by Cells in the Primary Cultures

The above results confirmed that epithelial cells were present in the cultures at least until day 4, but in the next experiment, I determined the proportion of epithelial cells present by staining with an epithelial cell specific anti-cytokeratin antibody. Primary cultures were harvested on days 1, 3 and 7 and stained for the presence of cytokeratin. Unfortunately, the photomicrographs of these results were destroyed by fire. However, this study showed that most of the cells in the day 1 cultures stained positively for cytokeratin which was present in the cytoplasm of the cells. However, with time, the proportion of cells positively staining cells in the cultures decreased.

Expression of Digestive Enzymes by Epithelial Cells in the Primary Cultures

It appeared from the above results that the primary cultures contained epithelial cells, many of which had a brushborder of microvilli similar to that seen *in vivo*. Thus, it was likely that these cells would express digestive enzymes and I therefore examined this directly. The primary cultures were harvested on days 1, 3 and 5 and stained for the presence of aminopeptidase, sucrase and lactase. Normal rat small intestine showed the expected expression of all three enzymes on the apical surface of the villus epithelial cells (Fig. 18, 19 and 20), while the secondary antibody alone showed no staining (Fig. 16). Secondary antibody alone also showed no staining on day 5 primary cultures (Fig. 17).

On day 1, primary cultures showed islands of cells which stained strongly for sucrase, aminopeptidase and lactase (Fig. 21, 22 and 23). The very bright positively stained centres of the islands contained cells which had not yet attached and spread on to the plastic. Virtually all the cells in the cultures stained positively for all 3 antibodies.

By day 3, all 3 enzymes were still expressed, but by now, staining was in many areas weaker and populations of cells appeared in the cultures which were negative for the 3 antibodies, and in particular these were the non-epithelial looking cells (Fig. 24, 25 and 26) (Unstained areas are not shown).

On day 5 cultures much larger areas of cells stained negatively for the 3 antibodies and these areas included the epithelial appearing cells. However, there were some positive areas which stained weakly positive with sucrase and lactase (Figs. 27 and 29), although cultures stained for aminopeptidase were virtually all negative (Fig. 28).

The expression of digestive enzymes by cells in the primary cultures confirmed the presence of epithelial cells, particularly at the beginning of the culture, when virtually all the cells stained positively.

The Effects of Growth Factors and Cytokines on the Growth of the Primary Cultures

As the primary cultures clearly contained epithelial cells, I proceeded to see if their growth could be influenced by any of the mediators which I had found to affect RIE-1 cells. Initially, I looked at the effect of EGF and as the culture medium used for the primary cultures contained 20ng/ml of EGF I examined whether reducing

concentrations to 10, 2 or 0ng/ml of EGF would alter epithelial cell growth. The amount of EGF in the medium had no significant effect on the growth of the cells on days 3 and 4 of culture. On day 7 of the experiment shown in Fig. 31, 20ng/ml EGF caused a significant increase in cell number compared with cultures without EGF but this was not observed on repeating the experiment. These results indicate that EGF has no significant effect on the growth of the primary cultures.

As IFNγ had the most potent effect on RIE-1 cells of all the cytokines examined, I next investigated whether IFNγ could have any effect on the growth of primary cultures. However, 100U/ml IFNγ, which had marked cytostatic effects on RIE-1 cells, had no significant effect on the growth of the cultures (Fig. 31).

Finally I examined whether supernatants from ConA-stimulated MLN cells would affect the growth of the primary cultures. Once again, addition of 1-10μl of activated supernatant had no significant effect on the growth of the cultures compared with controls (Fig. 32).

Conclusions

In this chapter I attempted to establish a primary model which could be used to assess the effects of cytokines on the growth and differentiation of normal epithelial cells. Initially, I confirmed that intact villus/crypt units isolated from neonatal rat intestine would grow in culture until confluence and that this growth could be measured by the uptake of crystal violet. Light microscopy showed that in the first 1-2 days, the cultures appeared to consist almost entirely of tightly packed polygonal epithelial cells. However, with time, other non-epithelial cell types began to appear. These cells

seem likely to be fibroblasts or smooth muscle cells. These findings were confirmed by the fact that in early cultures, virtually all cells in culture stained positive for cytokeratin, but as time progressed, the proportion of cytokeratin positive cells decreased.

Electron microscopic analysis of early primary cultures confirmed the presence of epithelial cells. Although these were more flattened than enterocytes *in vivo*, they had characteristic tight junctions and desmosomes and a brush border of microvilli. The epithelial cells were more differentiated in morphology than the RIE-1 cell line but appeared less differentiated than *in vivo* enterocytes. Confirming light microscopy and cytokeratin results, at later times other cells appeared which were completely flat and lacked microvilli or desmosomes, indicating that the cultures were not purely epithelial. Many of the more differentiated epithelial cells in the culture did not appear healthy and it is possible that these cells were not growing at all, but just surviving, before degenerating and dying after a few days.

Similarly, early cultures expressed large amounts of the digestive enzymes aminopeptidase, lactase and sucrase and this staining decreased as culture proceeded. Together, these findings indicated that although primary cultures of differentiated enterocytes could be established, these contained other cell types and mature enterocytes could not be observed after 4 days. This could be due to complete takeover by non-epithelial cells, or because only undifferentiated epithelial cells remained.

A high proportion of the cells in the primary culture were epithelial in origin (at least at the beginning of culture) and therefore, I examined the effects of the mediators EGF, IFNγ and MLN cell

supernatants on the growth of these cultures with the aim of comparing this with assays using RIE-1 cells. However, none of the mediators used had any effect on the growth of the cultures.

Thus, contrary to my expectations, the method used here did not result in primary cultures of pure small intestinal epithelial cells and was not appropriate for assessing the effects of immune mediators. Further work would be required to determine the exact composition and growth requirements before this system could be used.

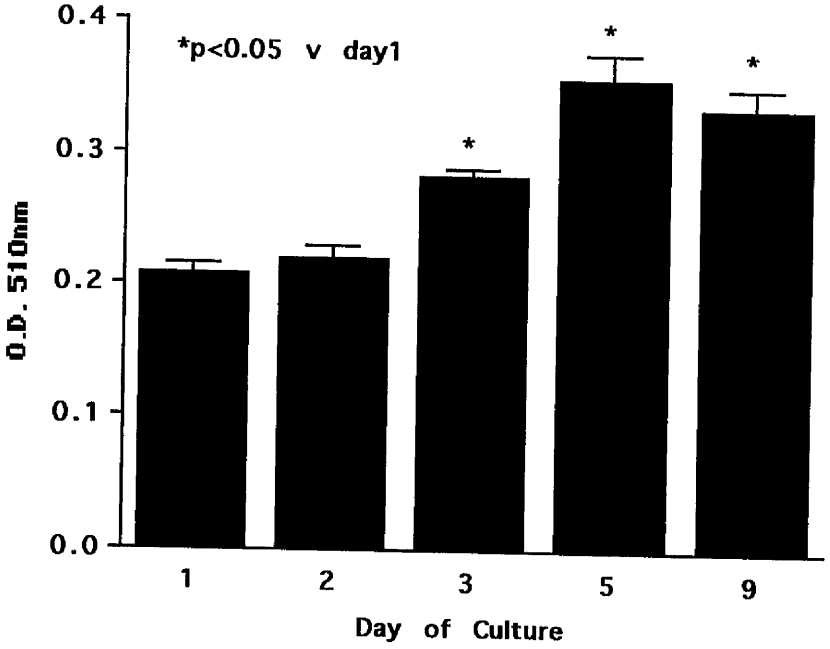

Figure 1: Time course of the growth of primary culture cells as assessed by crystal violet assay. Results shown are O.D/well+1s.e for quadruplicate assays.

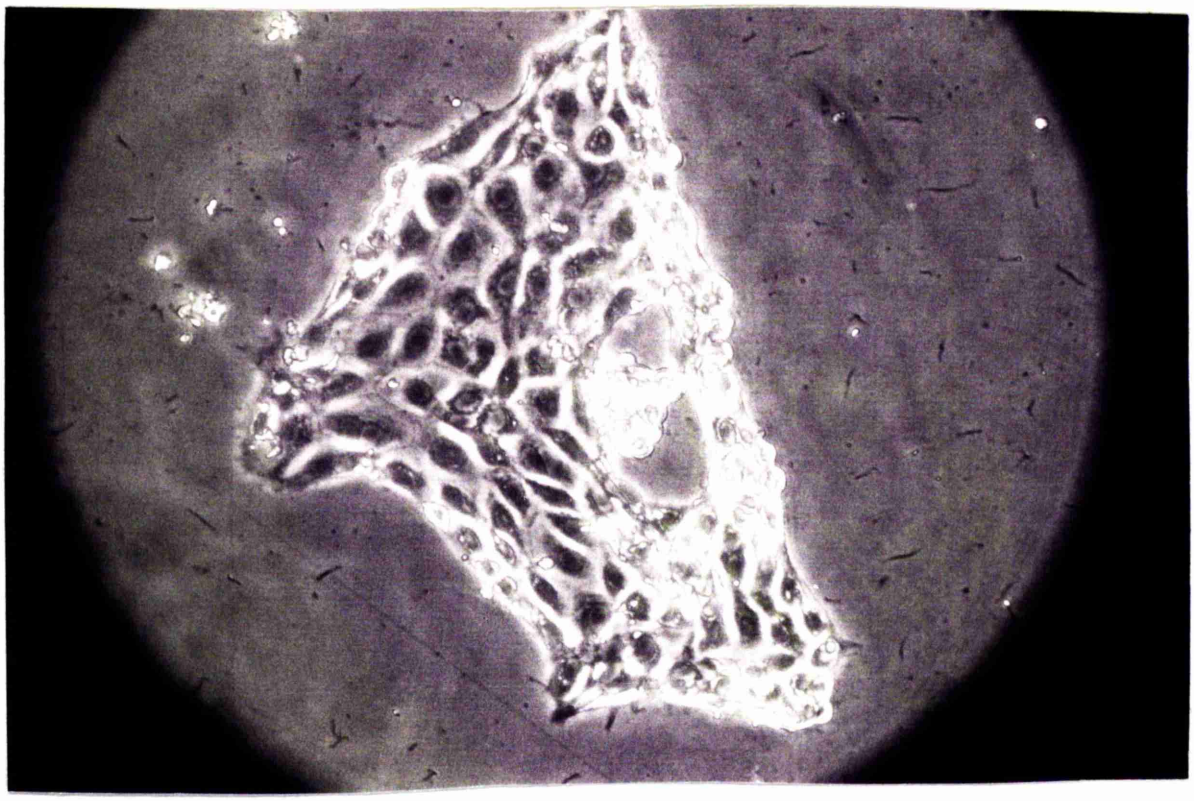

Figure 2: Day 1 primary culture showing an island of tightly packed epithelial cells derived from a single crypt/villus unit (x40).

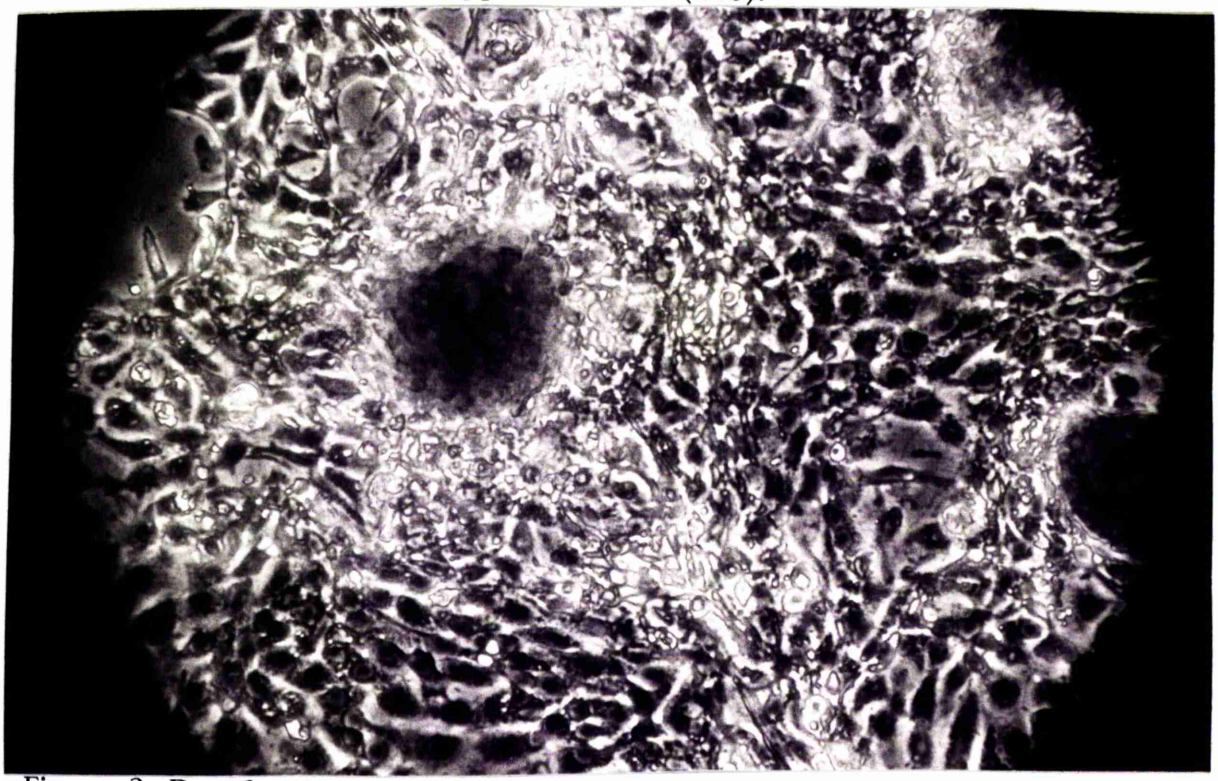

Figure 3: Day 6 primary culture showing central area of the well containing a sheet of tightly packed poygonal cells, resembling epithelial cells (x40).

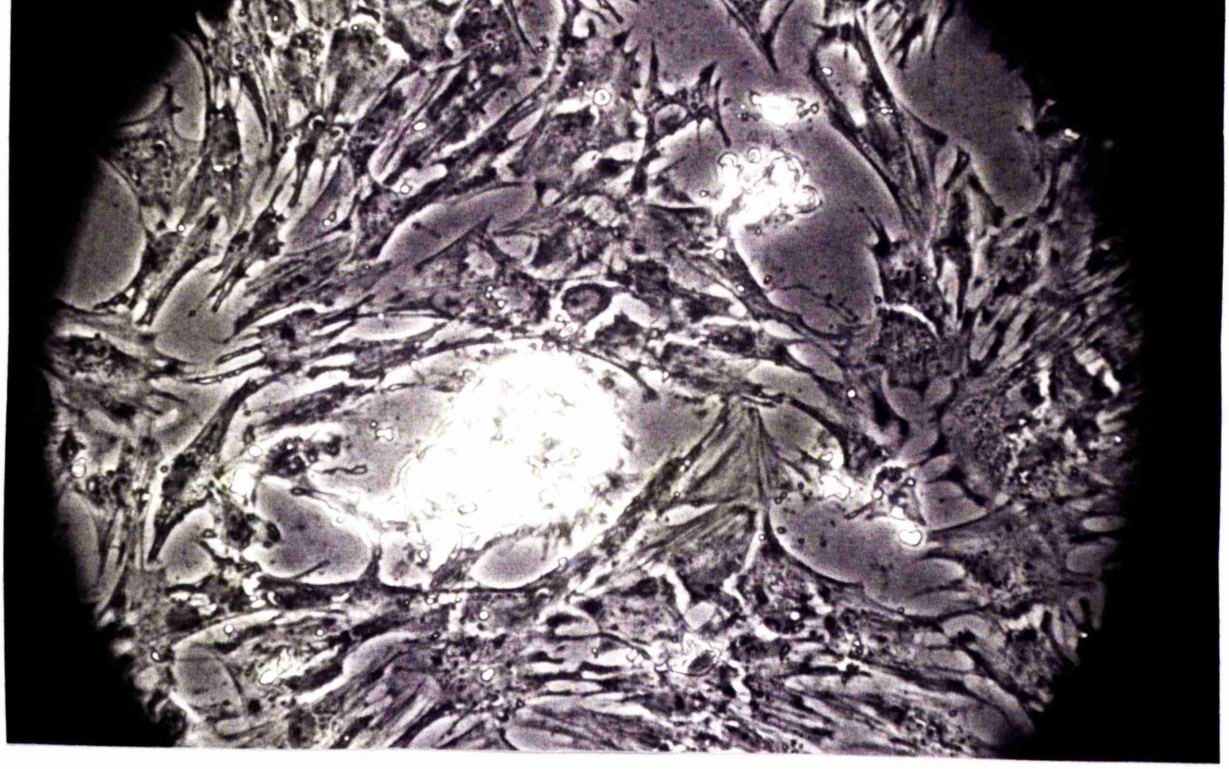

Figure 4: Day 6 primary culture showing an area of cells towards the outer edge of the well which are non-epithelial in appearance (x40).

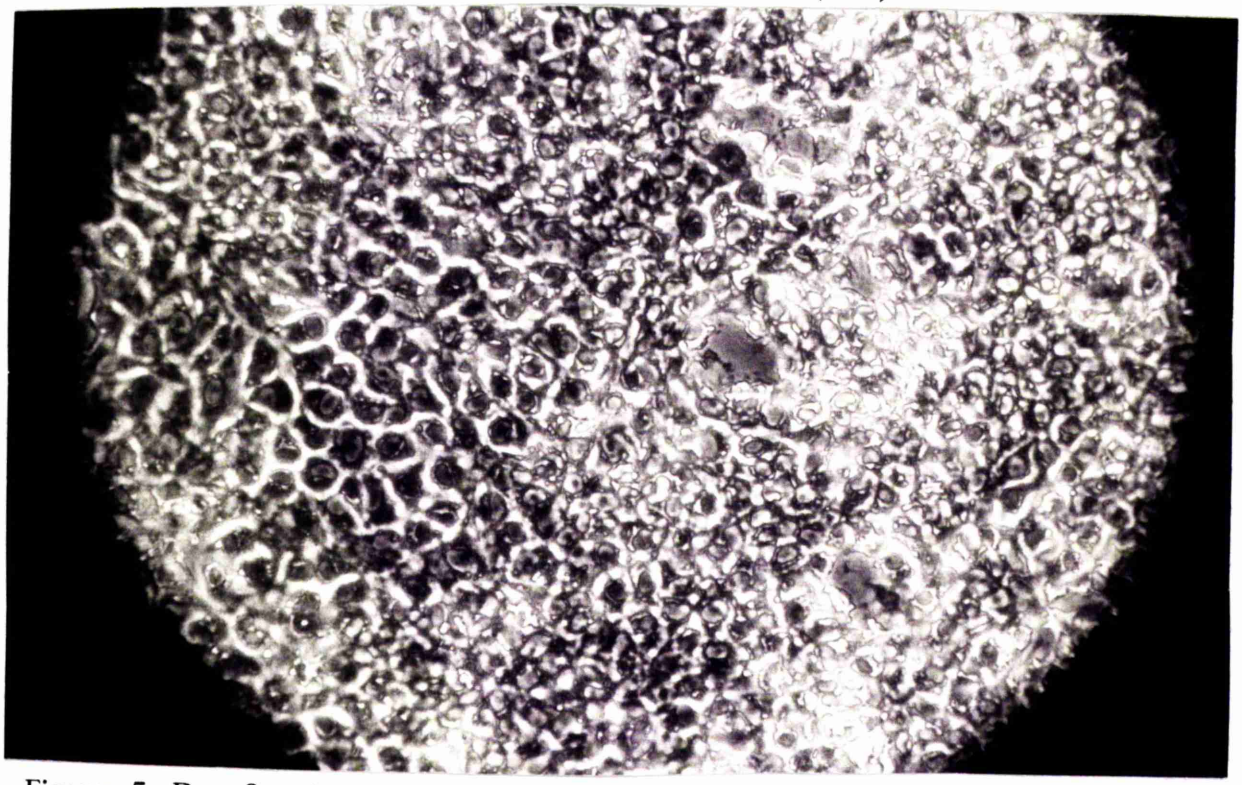

Figure 5: Day 9 primary culture showing central area of the almost confluent well containing a sheet of tightly packed poygonal cells, resembling epithelial cells (x40).

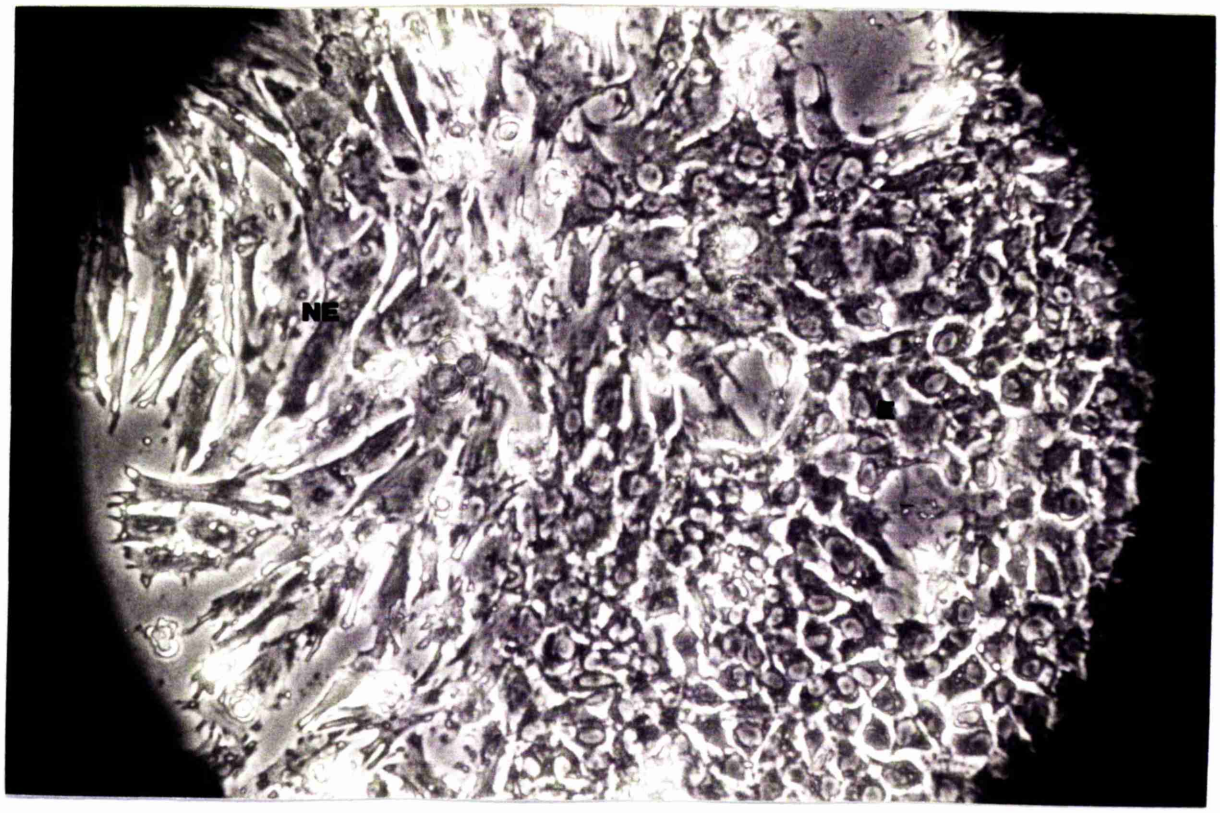

Figure 6: Day 9 primary culture showing an area of cells epithelial in appearance (E) in the right hand half of the well and an area of cells in the left hand half of the well which are non-epithelial in appearance (NE) (x40).

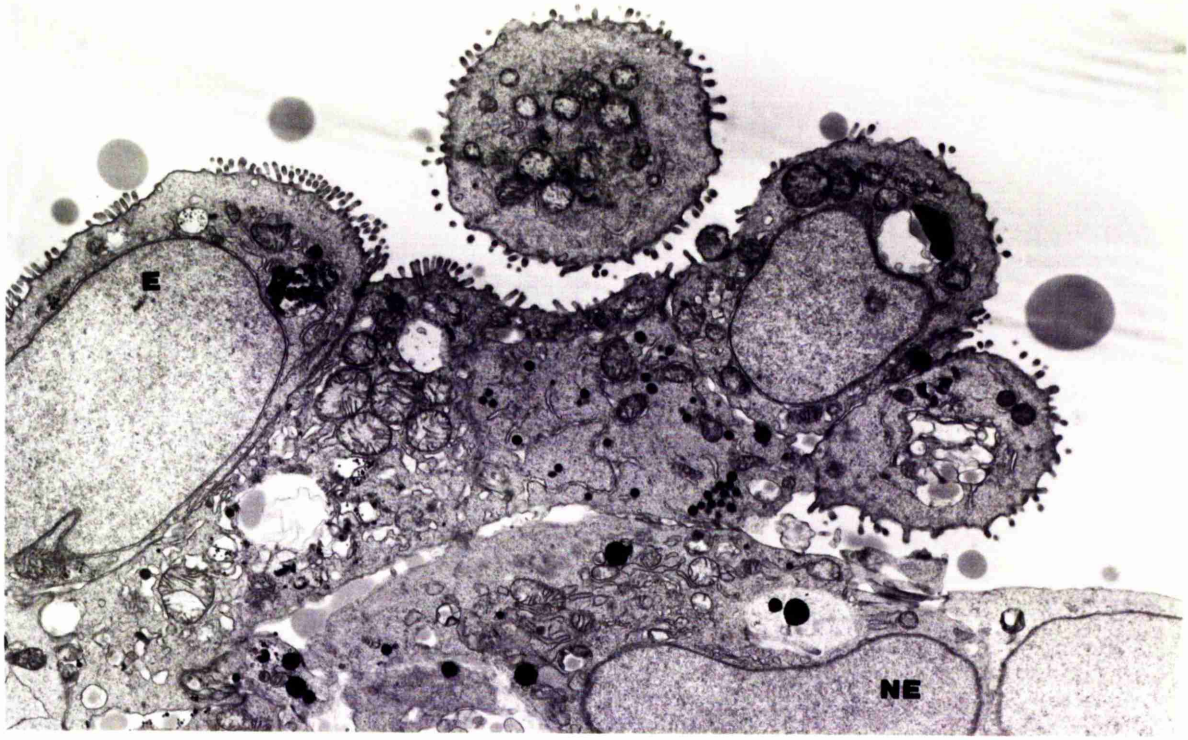

Fig. 7: Electron micrograph of a day 1 primary culture showing epithelial cells (E) with tight junctions and microvilli in the upper half of the picture and a layer of cells which are non-epithelial (NE) in appearance in the lower half (x4400).

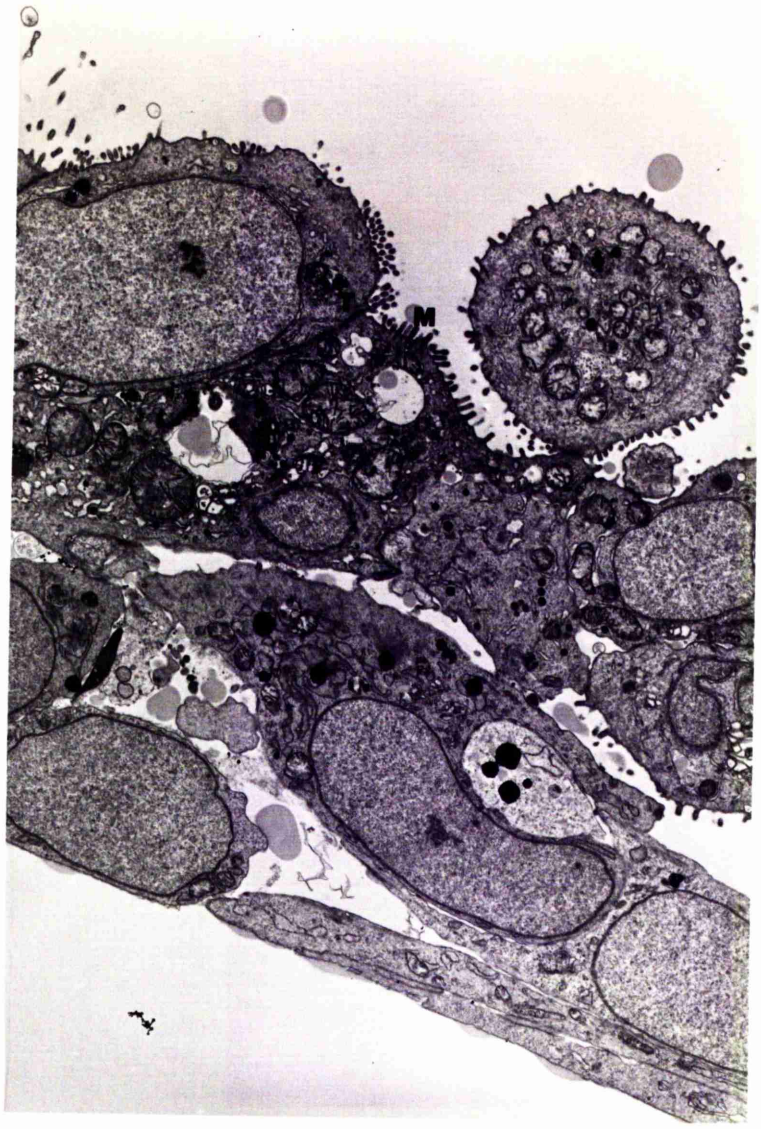

Fig. 8: Electron micrograph of a day 1 primary culture showing flattened cells some of which can be identified as epithelial due to the presence of tight junctions (T) and microvilli (M) (x7800).

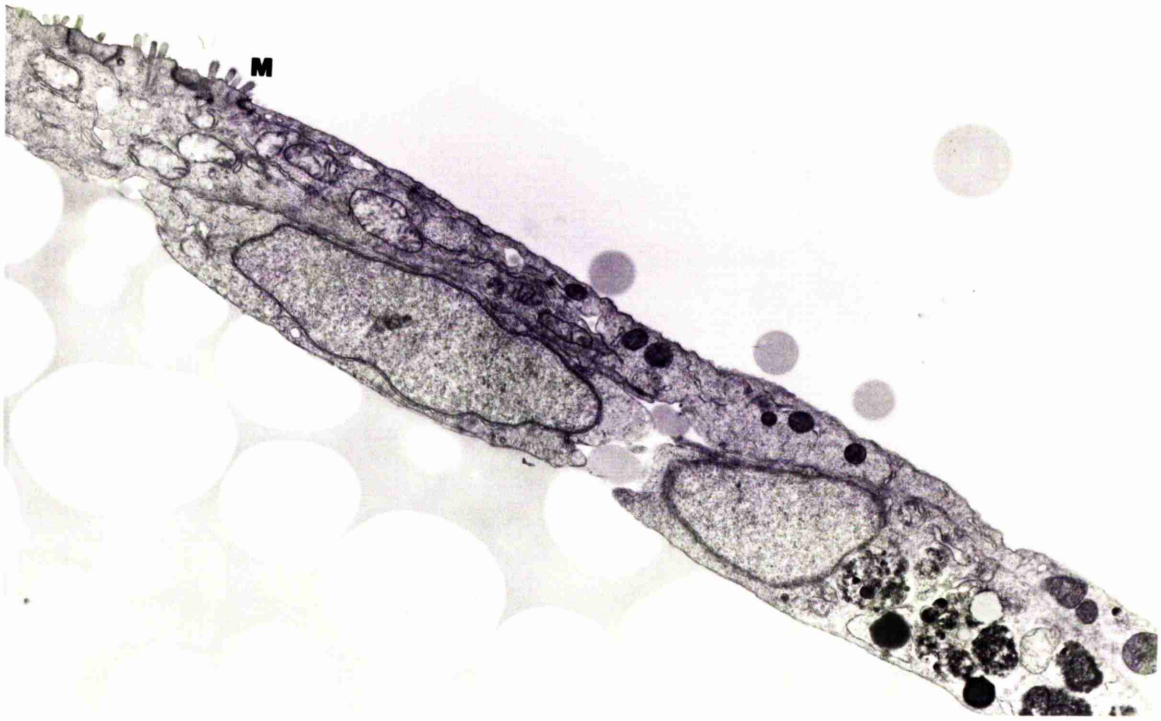

Fig. 9: Electron micrograph of a day 2 primary culture showing epithelial cells with microvilli (M) (x3300)

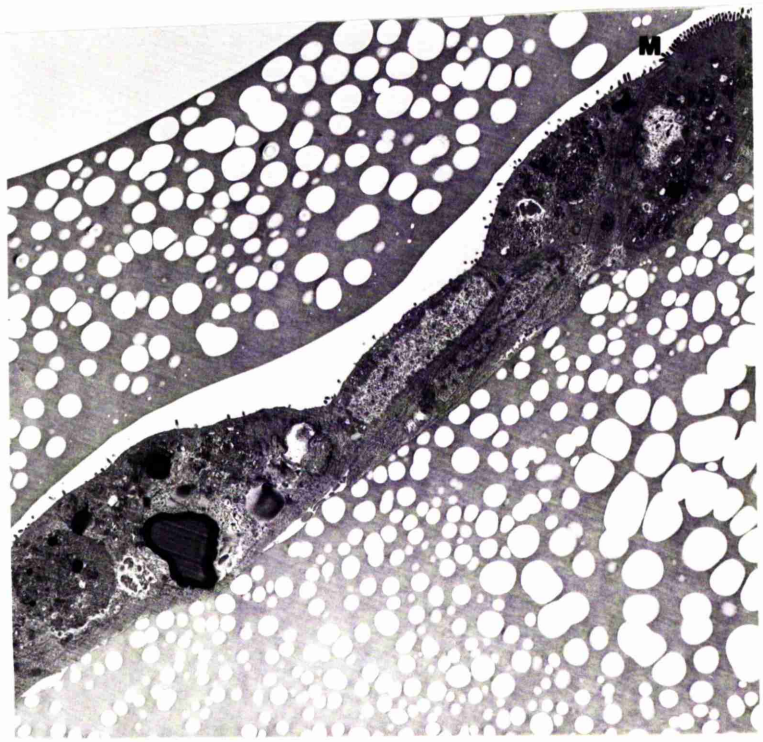

Fig. 10: Electron micrograph of a day 2 primary culture showing the presence of epithelial cells with microvilli (M) (x1400)

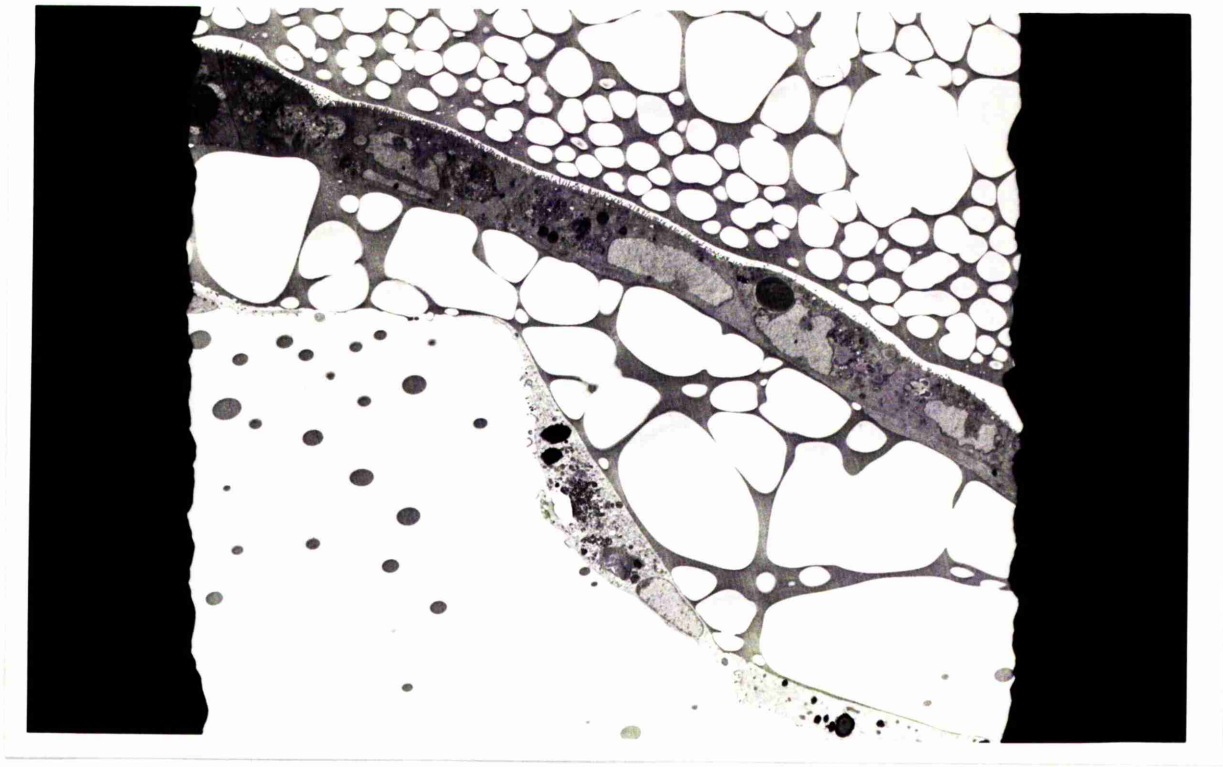

Fig. 11: Electron micrograph of a day 3 primary culture showing the presence of epithelial cells with microvilli (x1400).

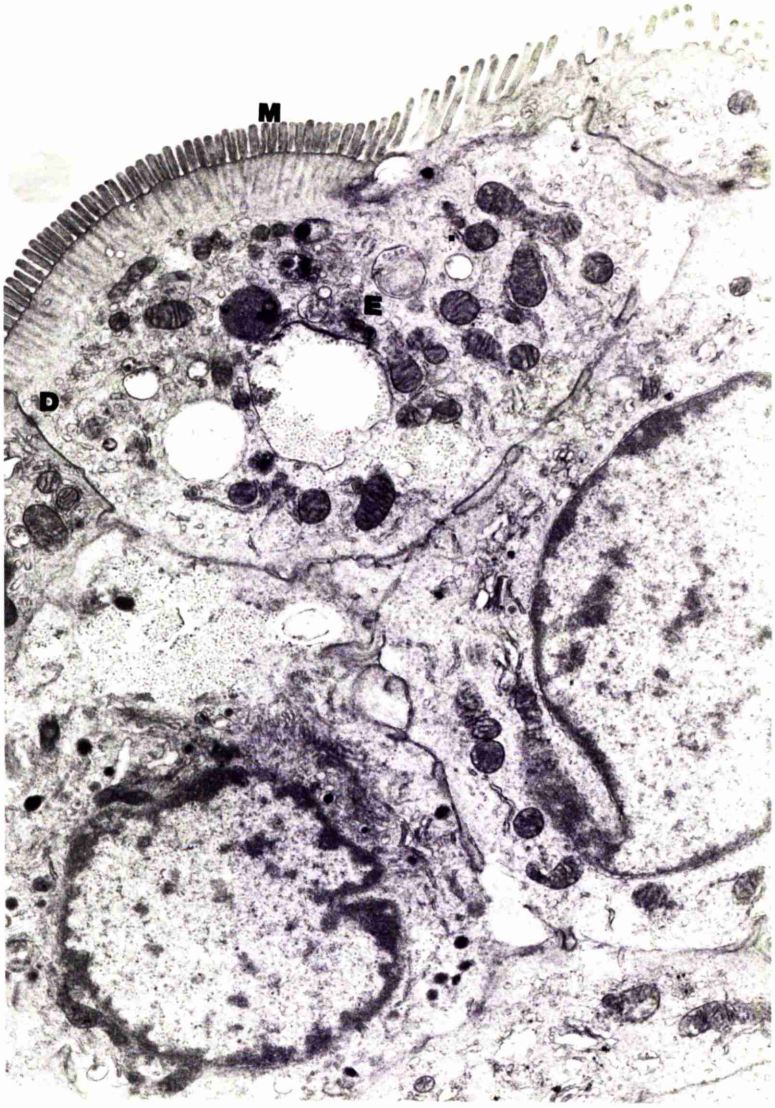

Fig. 12: Electron micrograph of a day 3 primary culture showing an epithelial cell (E) with well defined microvilli (M) and desmosomes (D) (x10400).

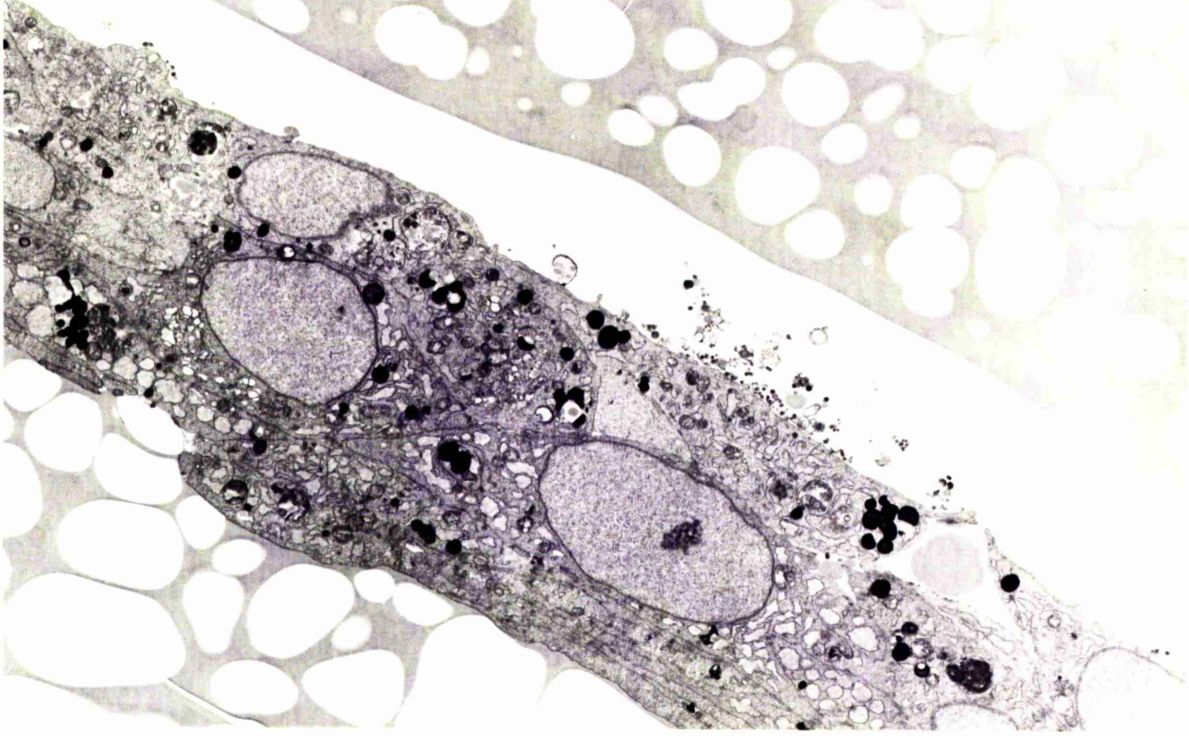

Fig. 13: Electron micrograph of a day 4 primary culture showing a multilayered structure with no evidence of epithelial cells (x3300)

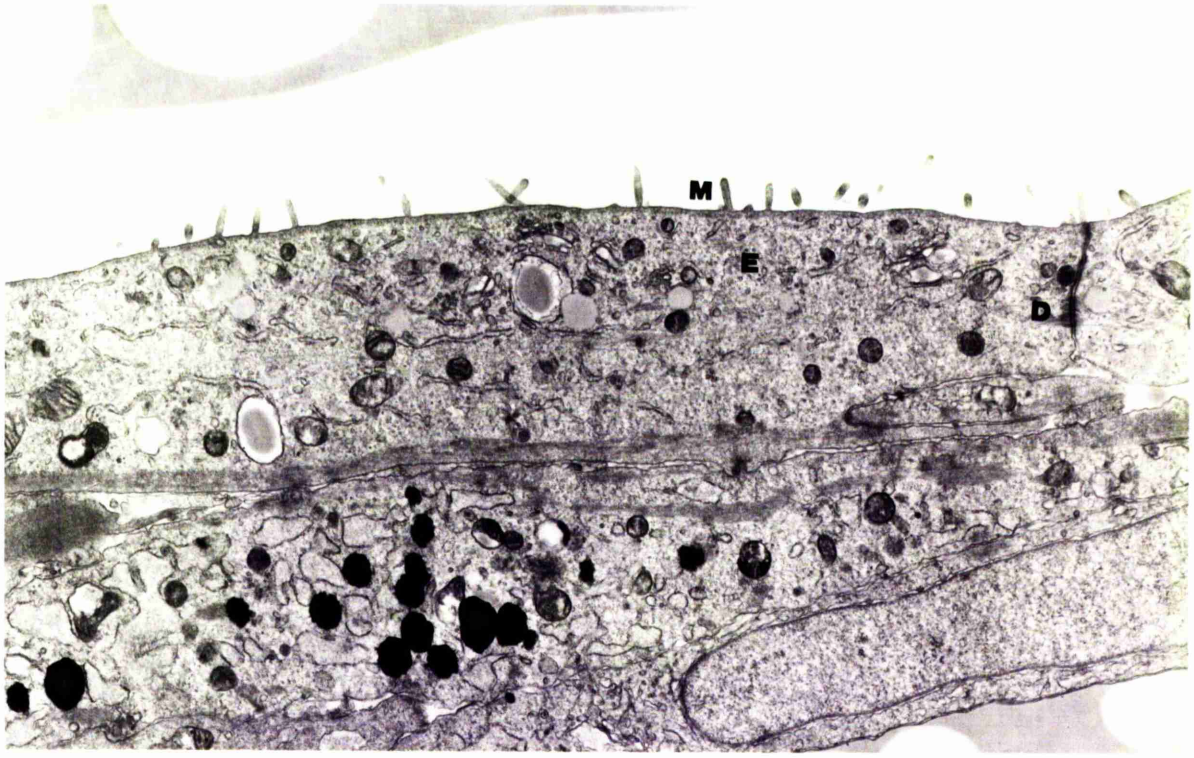

Fig. 14: Electron micrograph of a day 4 primary culture showing a flattened epithelial cell (E) with microvilli (M) and desmosomes (D) on the upper layer (x3300).

Fig. 15: Unstained tissue section of rat small intestine (x10).

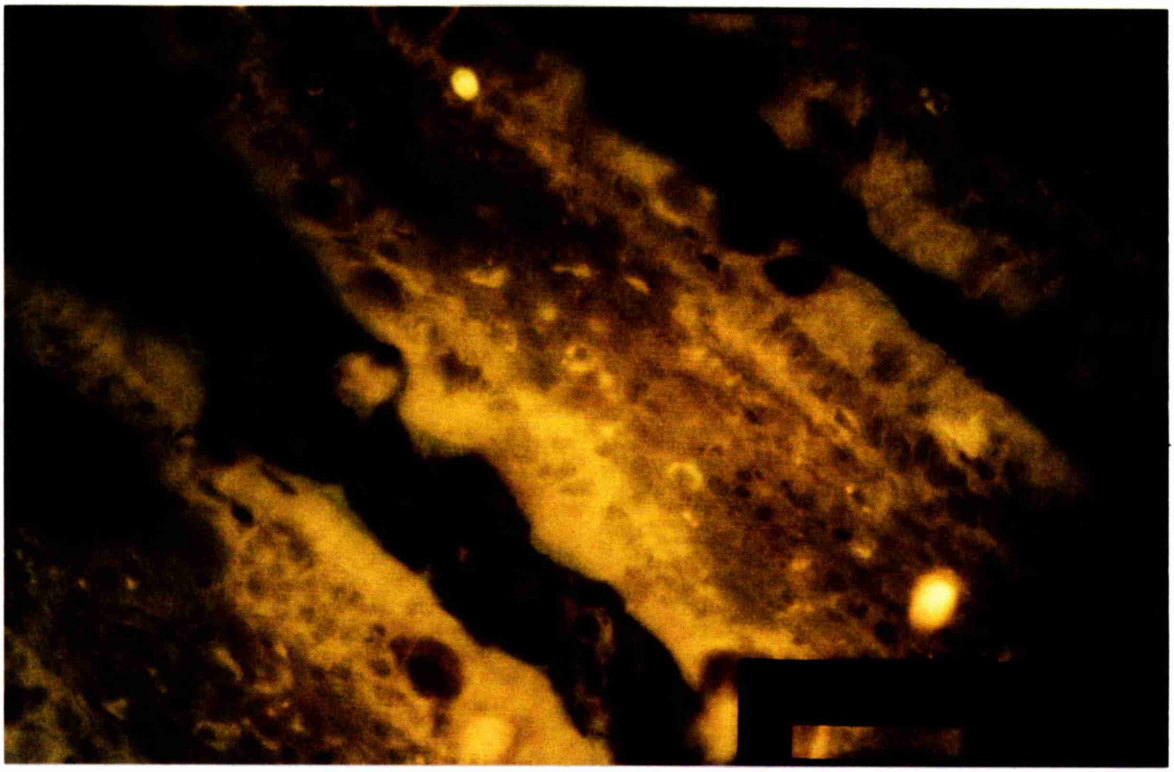

Fig. 16: Tissue section of rat small intestine showing negative staining of villi with secondary antibody only (x40)

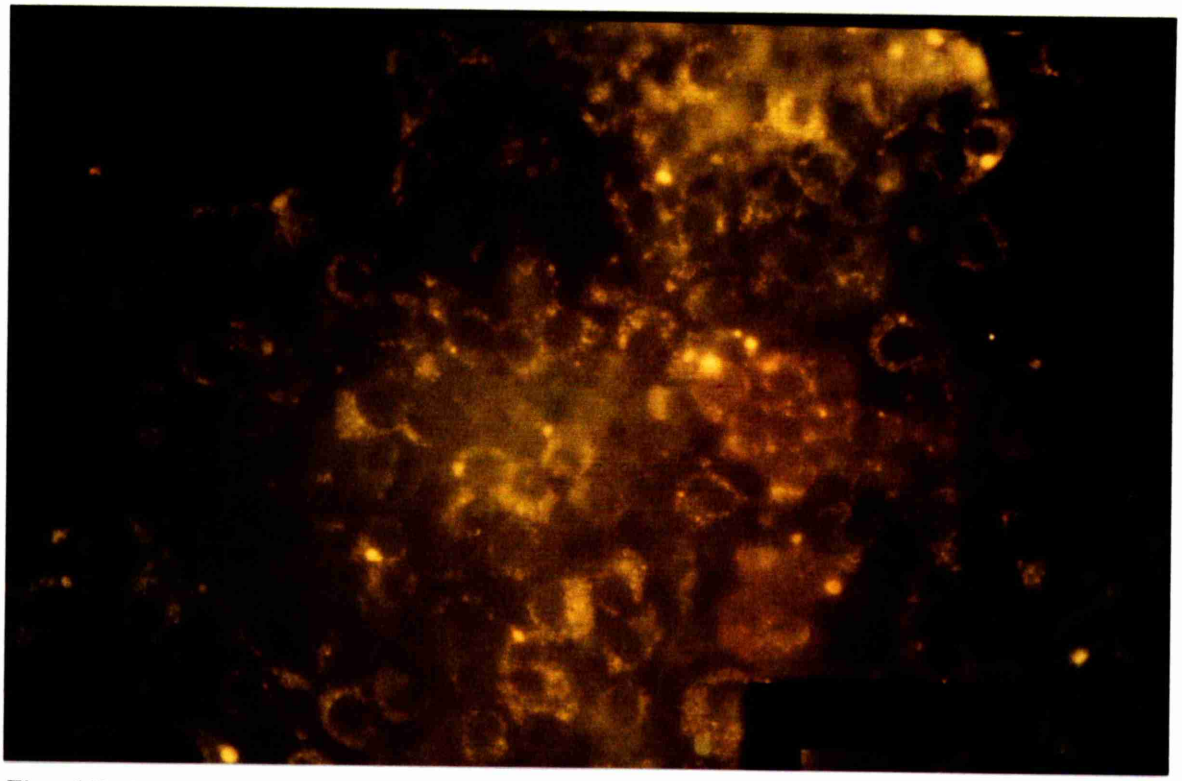

Fig. 17. Microscopic appearance of a 5 day primary culture of small intestinal cells stained with secondary antibody only and showing negative staining. (x40)

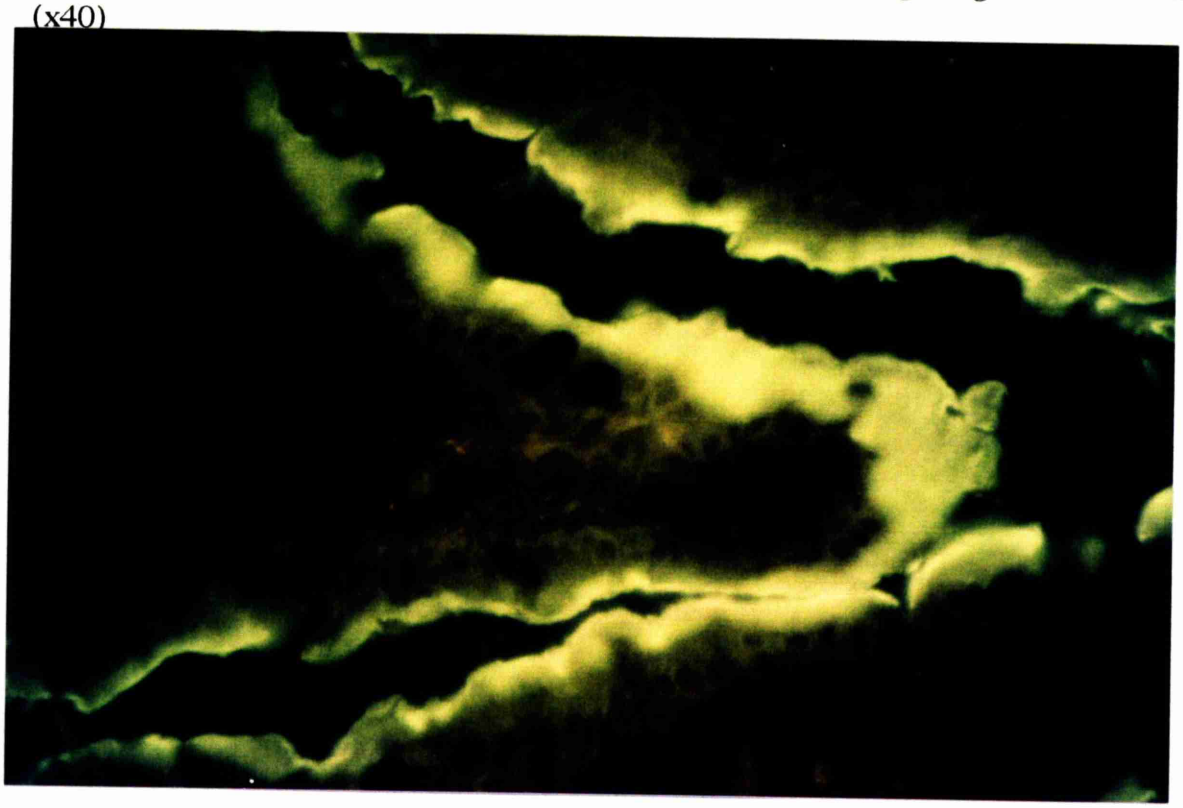

Fig. 18: Microscopic appearance of a tissue section of rat small intestine stained for sucrase expression. The apical surface of the epithelial cells on the villi stain strongly positive. (x40)

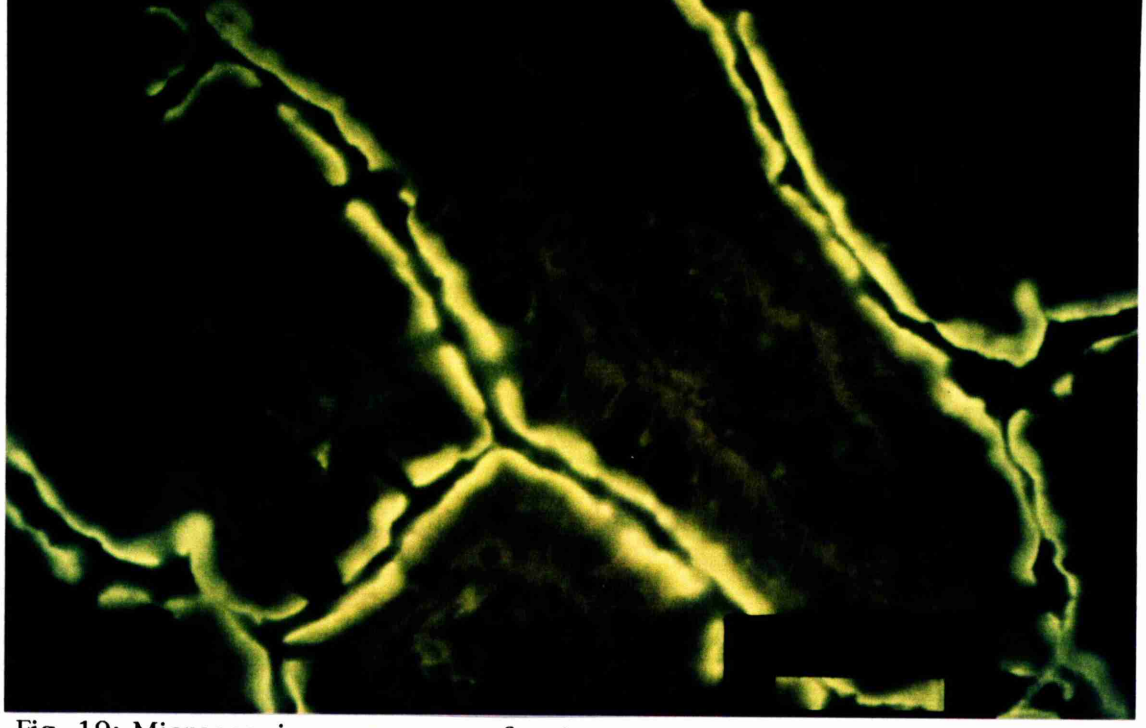

Fig. 19: Microscopic appearance of a tissue section of rat small intestine stained for aminopeptidase expression. The apical surface of the epithelial cells on the villi stain strongly positive. (x40)

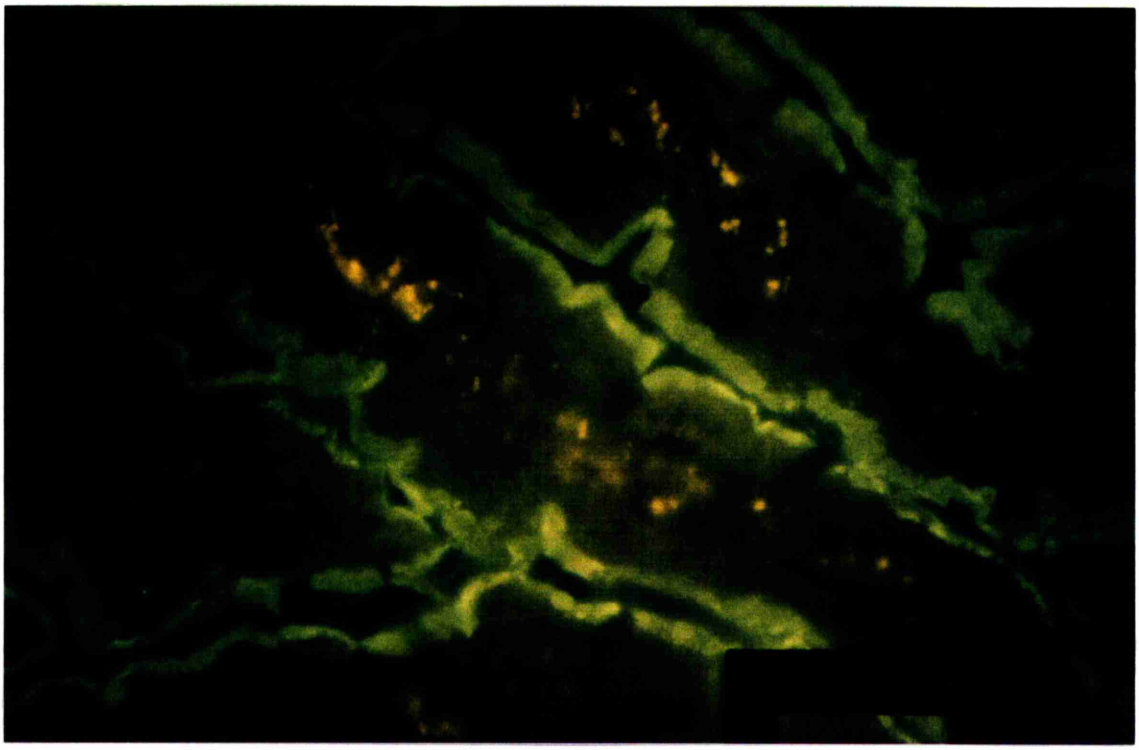

Fig. 20: Microscopic appearance of a tissue section of rat small intestine stained for lactase expression. The apical surface of the epithelial cells on the villi stain strongly positive. (x40)

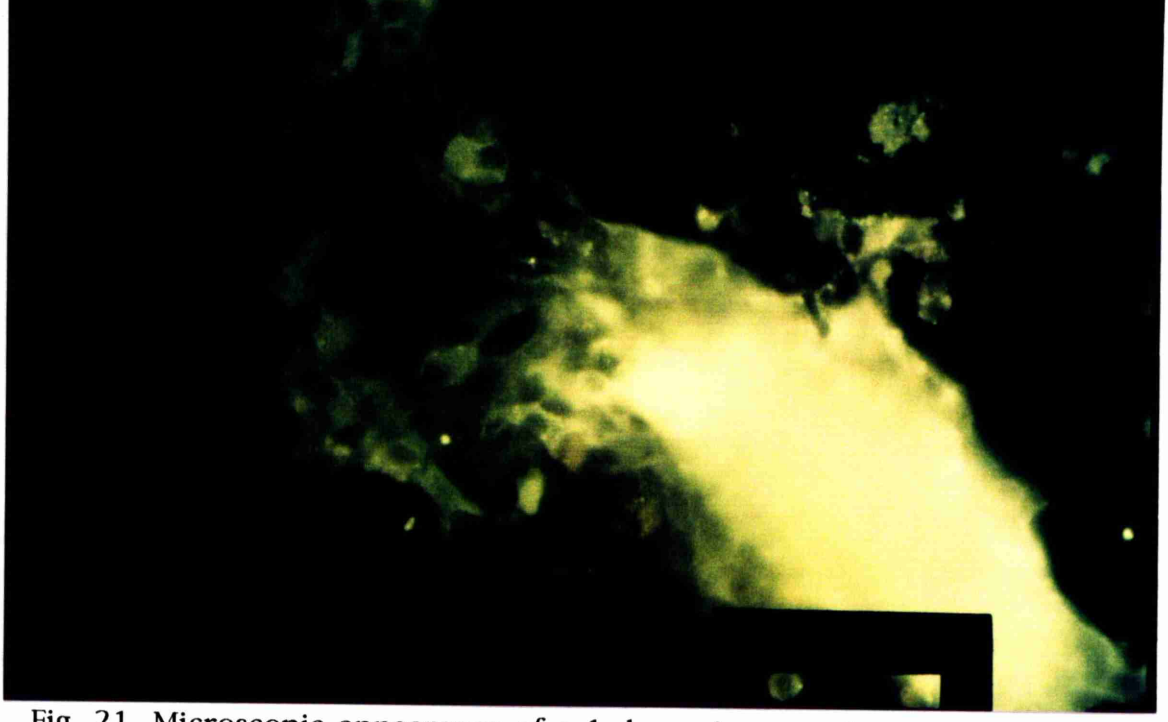

Fig. 21. Microscopic appearance of a 1 day primary culture of small intestinal cells stained for sucrase expression. The island of cells stains very strongly positive. The very brightly stained centre areas contained unattached cells of the villus/crypt unit (x40).

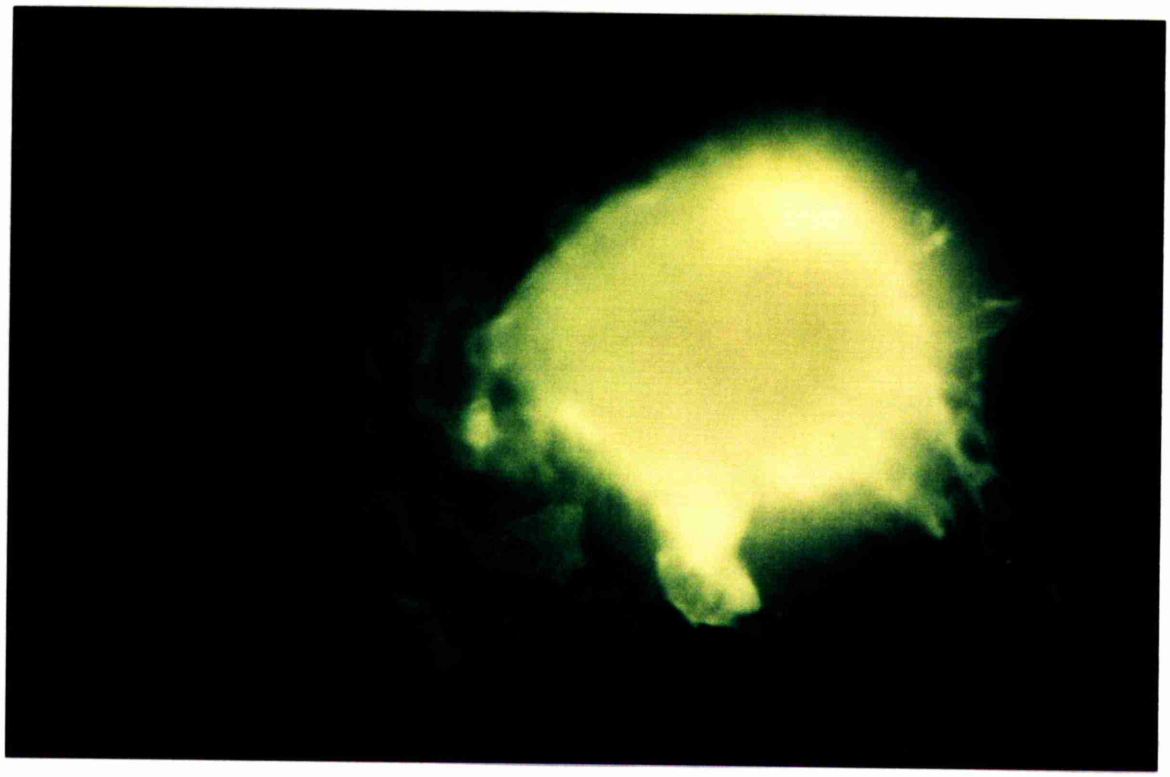

Fig. 22. Microscopic appearance of a 1 day primary culture of small intestinal cells stained for aminopeptidase expression. The island of cells stains very strongly positive. The very brightly stained centre areas contained unattached cells of the villus/crypt unit (x40)

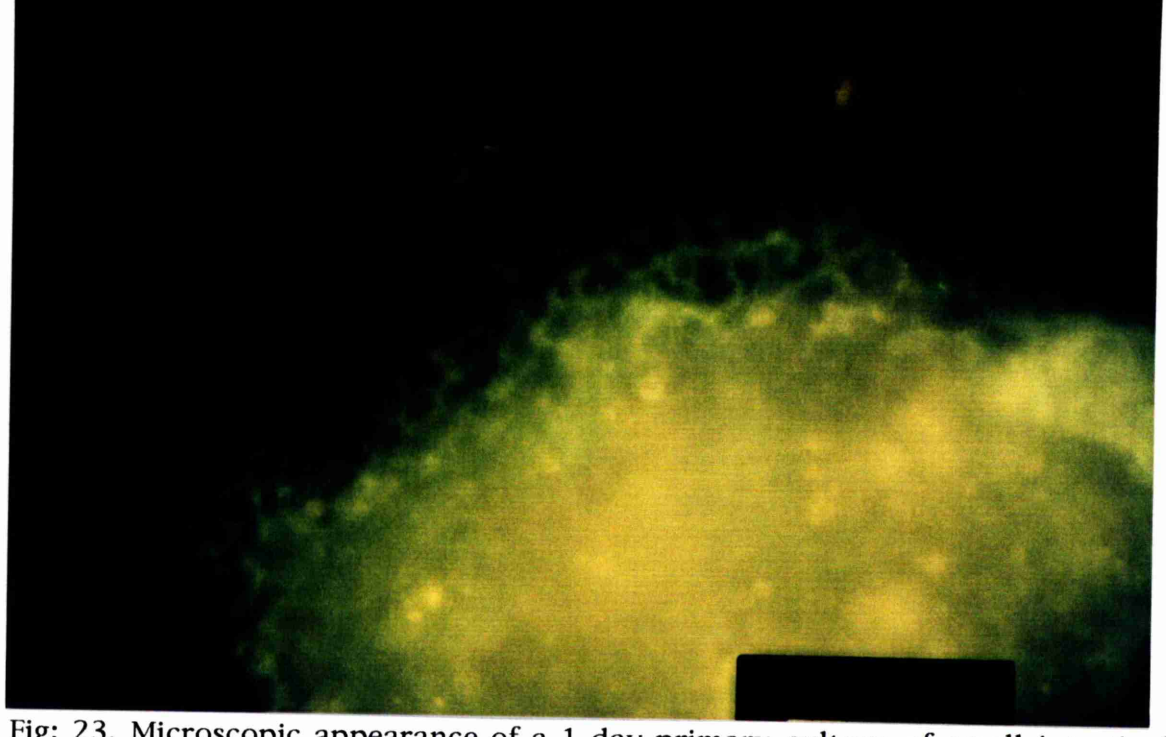

Fig: 23. Microscopic appearance of a 1 day primary culture of small intestinal cells stained for lactase expression. The island of cells stains very strongly positive. The very bright centre area contains unattached cells from the crypt/villus unit (x40)

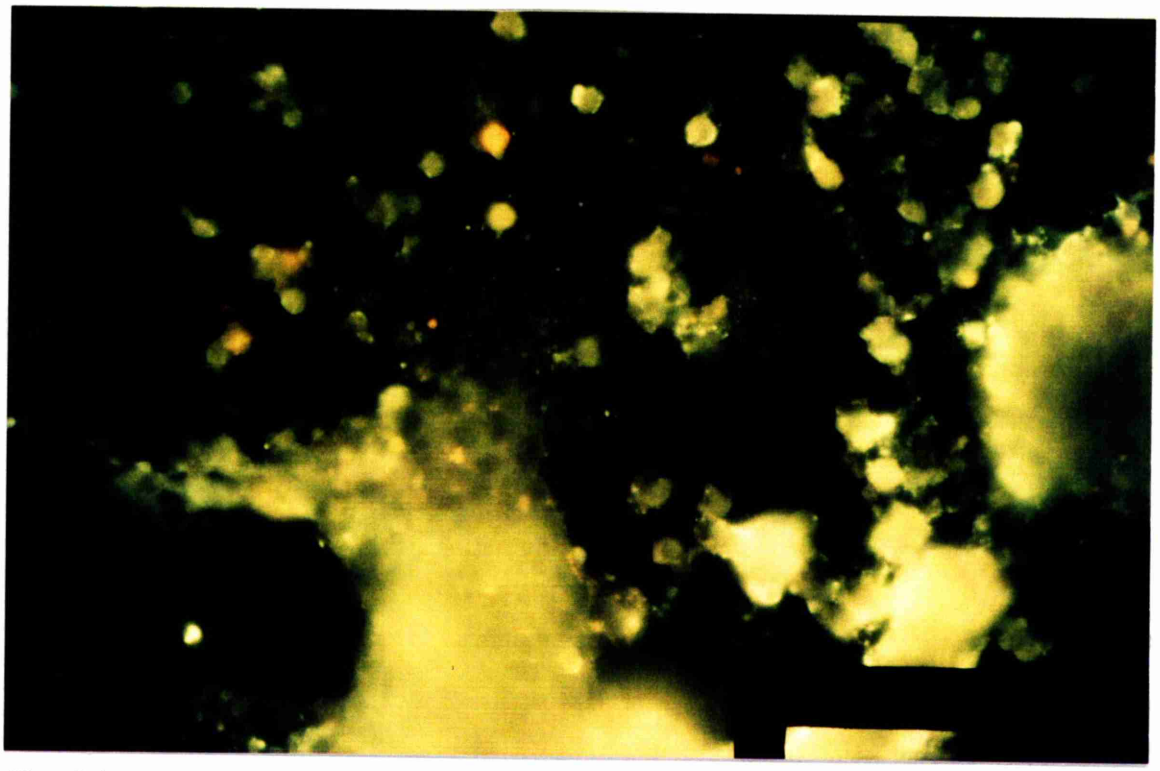

Fig: 24. Microscopic appearance of a 3 day primary culture of small intestinal cells stained for sucrase expression. Positive staining of the cell sheet. (x40)

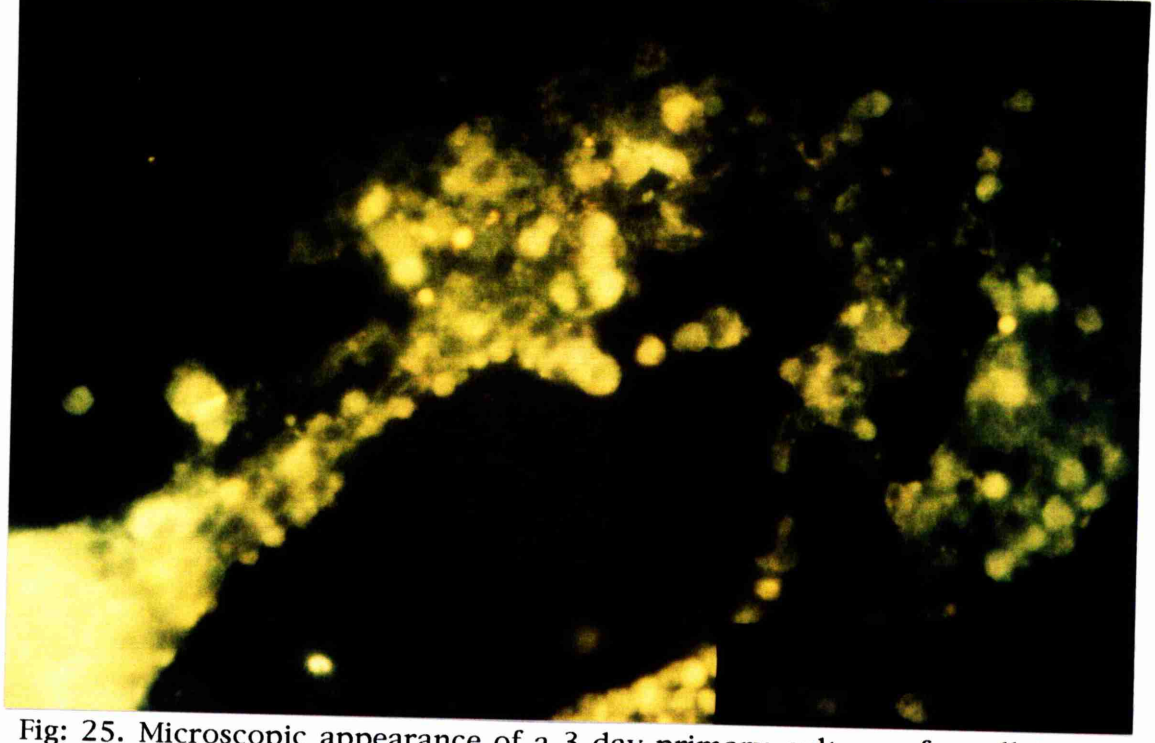

Fig: 25. Microscopic appearance of a 3 day primary culture of small intestinal cells stained for aminopeptidase expression. Positive staining of the cell sheet. (x40)

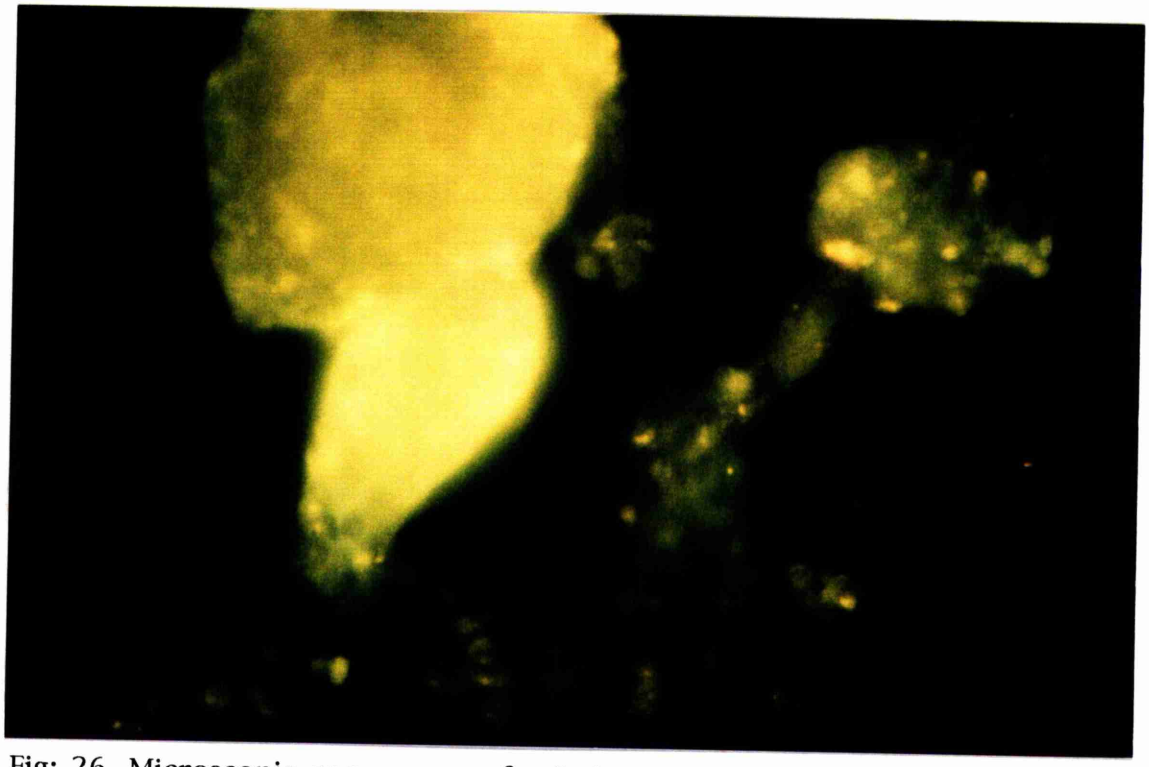

Fig: 26. Microscopic appearance of a 3 day primary culture of small intestinal cells stained for lactase expression. Positive staining of the cell sheet. (x40)

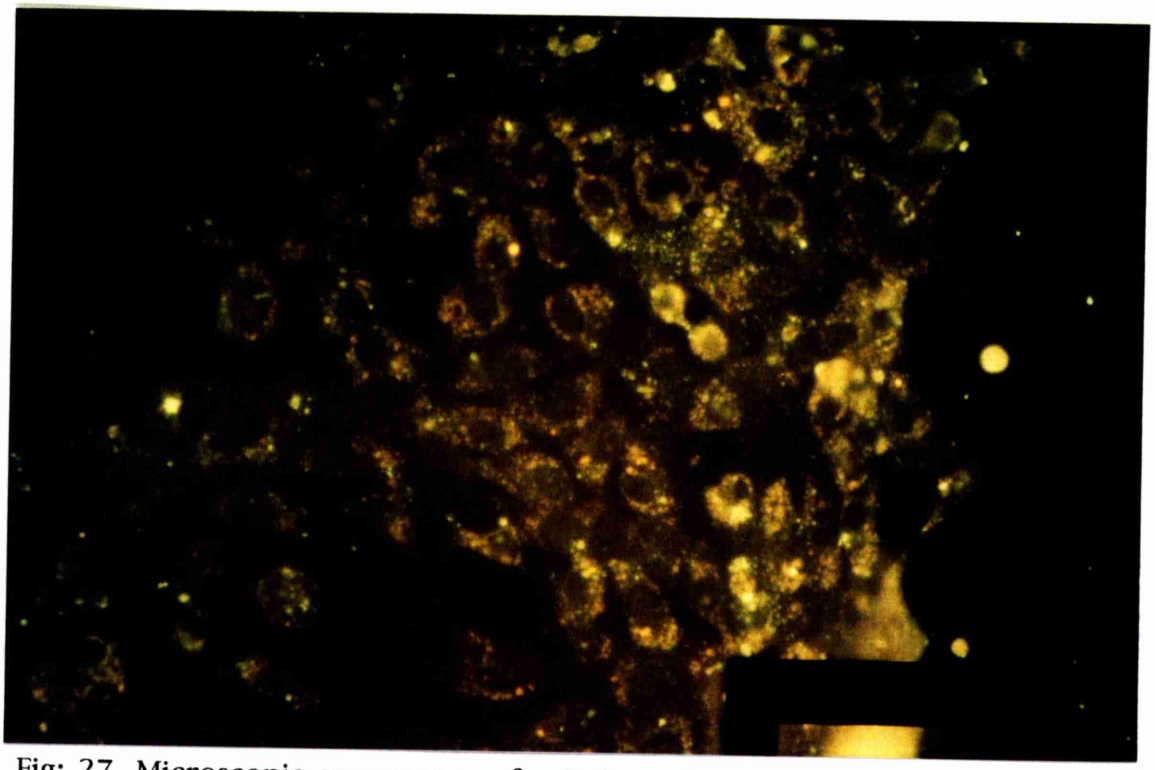

Fig: 27. Microscopic appearance of a 5 day primary culture of small intestinal cells stained for sucrase expression. Very weak positive staining of cells. (x40)

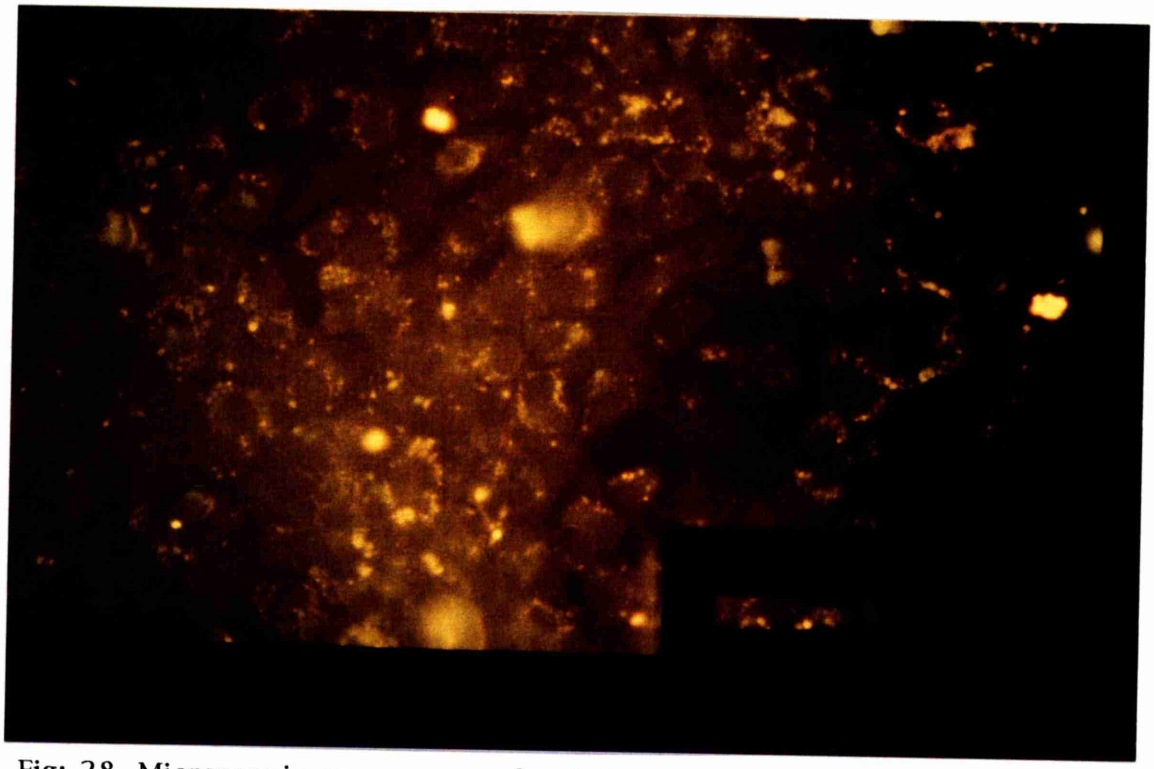

Fig: 28. Microscopic appearance of a 5 day primary culture of small intestinal cells stained for aminopeptidase expression. Virtually the whole area has stained negative. (x40)

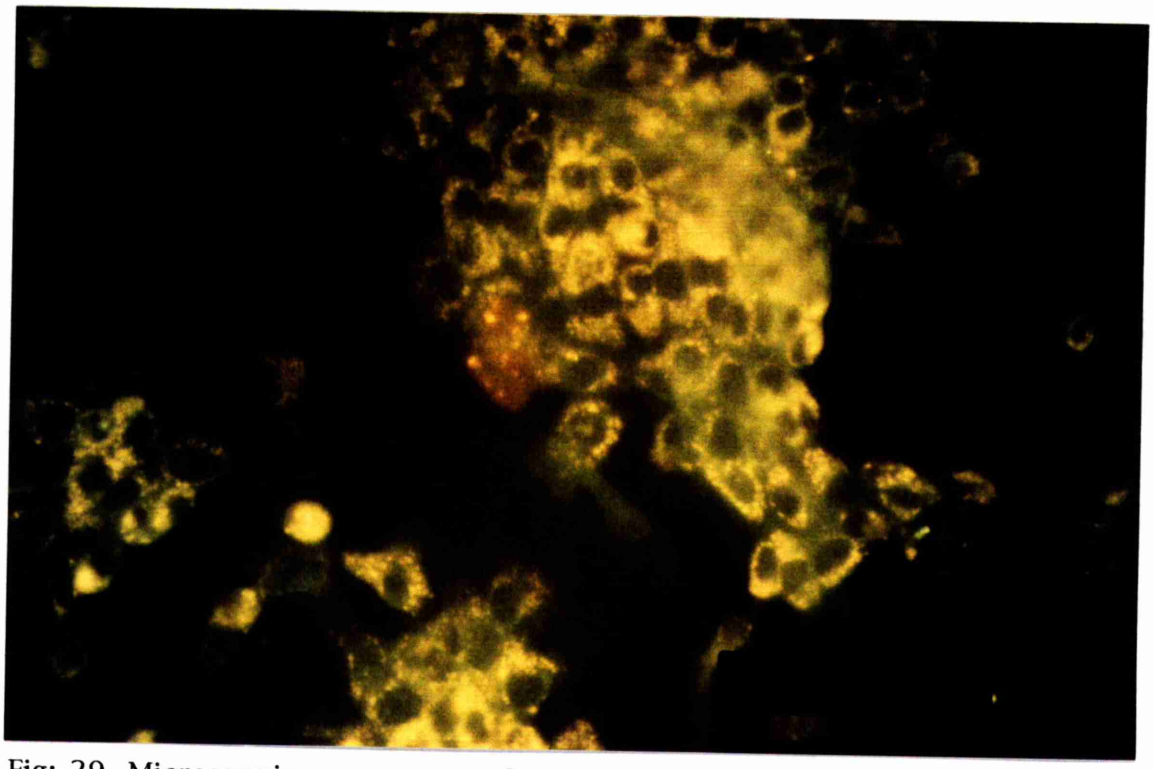

Fig: 29. Microscopic appearance of a 5 day primary culture of small intestinal cells stained for lactase expression. Weak positive staining of the cell sheet. (x40)

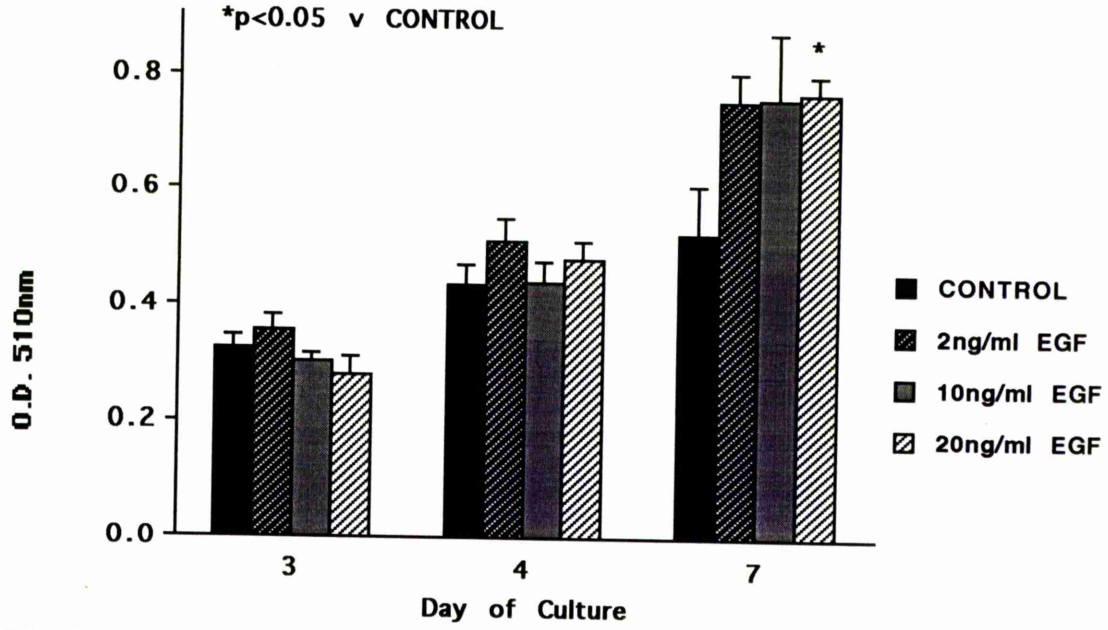

Fig 30: The effect of different concentrations of EGF on the growth of primary cultures of intestinal epithelial cells. Results shown are mean O.D/well+1s.e for quadruplicate assays.

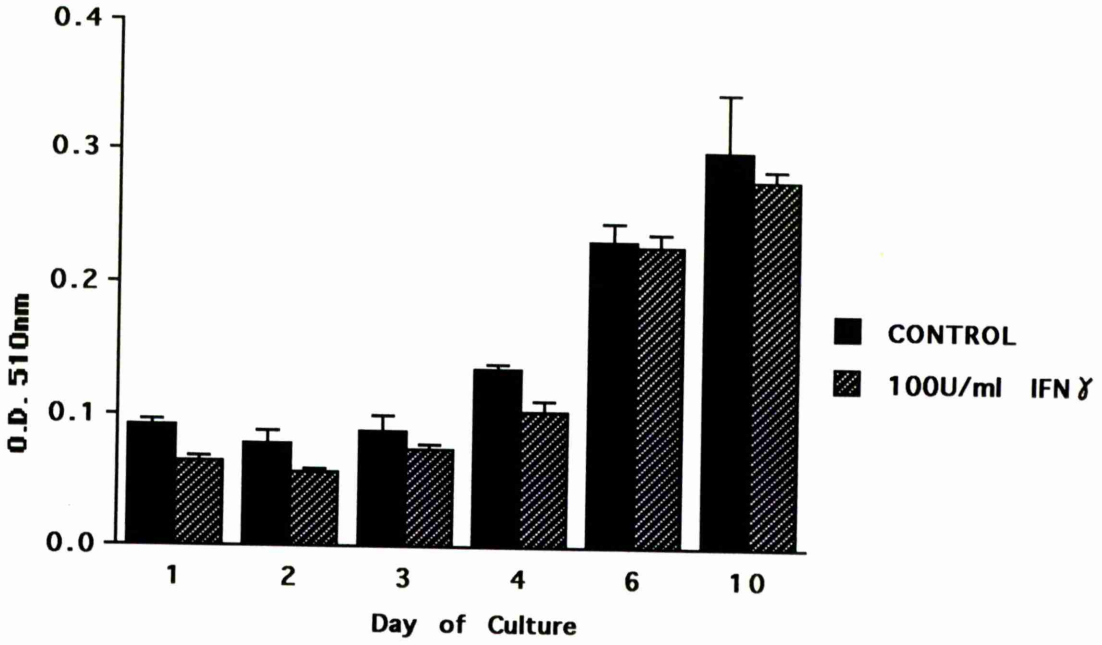

Fig 31: The effect of 100U/ml of IFNγ on the growth of primary cultures as assessed by a crystal violet assay. Results shown are mean O.D/well +1s.e. for quadruplicate assays.

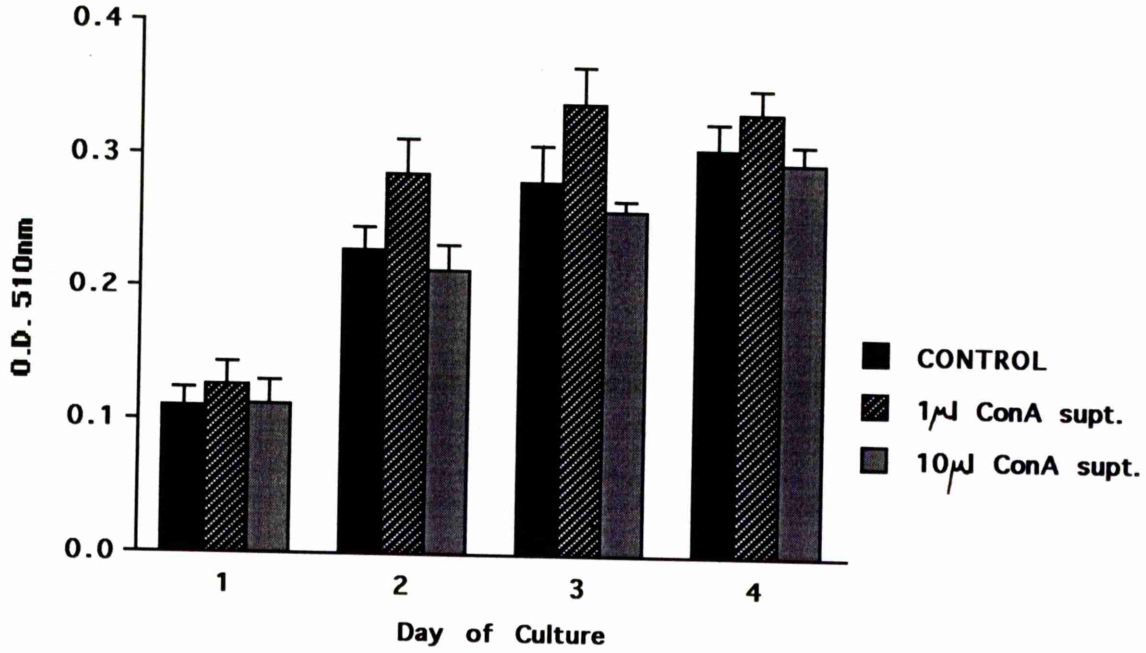

Fig 32: The effect of supernatant from ConA stimulated MLN cells on the growth of primary cultures as assessed by the crystal violet assay. Results shown are mean O.D/well+1s.e. for quadruplicate assays.

CHAPTER 9

GENERAL DISCUSSION

The aim of this thesis was to investigate the hypothesis that crypt hyperplasia in intestinal GvHR and related enteropathies is due to a direct effect of cytokines on crypt epithelial cells. As all the existing evidence for this idea is based on *in vivo* models or organ cultures where it is not possible to look at direct effects of cytokines on epithelial cells, my project was designed to establish *in vitro* models of dividing crypt epithelial cells in which the effects of cytokines on the growth and differentiation of enterocytes could be investigated. Much of the work in this thesis was carried out using the RIE-1 cell line as a model and, although I also attempted to establish primary cultures of intestinal epithelial cells, these did not yield conclusive results.

I thought it appropriate to use a cell line for my studies, as this provided a uniform population of epithelial cells which could be maintained in culture in the absence of other cell types. It was also anticipated that the uniform nature of the population would increase the reproducibility of quantitative assays. Initially, I identified five criteria which an optimal cell line model would have. First it would be derived from epithelial cells of the small intestine, preferably with the morphological characteristics of undifferentiated crypt epithelial cells and the ability to respond to physiological regulators of epithelial cell function. In addition, it was important that the cell line was non-transformed and non-tumorigenic and, ideally, it would have the capacity to differentiate.

At the time I began my study, a number of intestinal epithelial cell lines were available and had been used in similar work. However, many of these such as the HT-29 and Caco-2 cells were either tumour derived or transformed, and derived from the human colon

(Zweibaum et al., 1985, Pinto et al., 1983). In addition, Caco-2 cells have the unusual property of differentiating into small intestinal enterocytes. I decided it was important that any cell line I used was non-transformed, to avoid the possibility that any cytokine-mediated effects might be due to the tumorigenic nature of the cells, as has been described with the cytotoxic effects of TNFα (Wang et al., 1985).

The non-transformed epithelial cell lines which were available included the IEC-6 cell line and the RIE-1 cell line (Quaroni et al., 1979, Blay and Brown, 1984). Both were derived from the small intestine of rats by similar methods and both had epithelial cell characteristics such as expression of cytokeratin and positive staining with monoclonal antibodies specific for epithelial cells. These cell lines also had morphological characteristics of epithelial cells, growing readily as "cobblestone" monolayers in culture. As I had access to the RIE-1 cell line, I decided to assess its suitability for my studies. Initially, I confirmed its epithelial nature by showing the expression of acidic cytokeratins in the cytoplasm of the cells. Subsequent EM studies showed confluent RIE-1 cells have tight junctions and desmosomes, features which are unique to epithelial cells. Cultures of RIE-1 cells also had an undifferentiated appearance, being non-polarised without a brush border of mature microvilli. Although small microvillus-like projections were present on the surface of the cells, these were sparse and could not be conclusively identified. These projections were much shorter than microvilli and did not have a central bundle of filaments extending into the cytoplasm of the cells. Together with other reports that RIE-1 cells express a marker found only on crypt cells *in vivo* (Blay and Brown, 1984), these features suggested that RIE-1 cells might be suitable for my purposes.

To determine whether RIE-1 cells would also be influenced by physiological mediators in a manner characteristic of epithelial cells *in vivo*, I examined their response to EGF and TGFβ. EGF had a dose dependent stimulatory effect on RIE-1 cell growth, consistent with its effects on other epithelial cells (Rheinwald and Green, 1977, Challacombe and Wheeler, 1991). Conversely, TGFβ which has a growth inhibitory effect on cells of epithelial origin (Moses et al., 1990, Barnard et al., 1989, Kurokowa et al., 1987), markedly inhibited RIE-1 growth. These findings confirmed that the RIE-1 cell line might be a suitable model for studying the direct effects of cytokines on growth of crypt epithelial cells. As there was no published evidence on whether this cell line had the capacity to differentiate *in vitro*, I also attempted to induce differentiation using TGFβ, IFNγ and a fibroblast feeder layer, but these did not yield conclusive results. Neither TGFβ nor IFNγ had any effect on the morphology of the RIE-1 cells as assessed by light microscopy or EM, although IFNγ induced expression of MHC molecules which are associated with differentiation *in vivo* (Steiniger et al., 1989). Co-culture with a fibroblast monolayer caused dramatic changes in the morphology of the RIE-1 cells, but these changes were not entirely characteristic of differentiation. These results suggest that the RIE-1 cell line does not have the capacity to differentiate, possibly due to the fact that it has the capacity to divide continuously *in vitro* and differentiation is usually associated with cessation of cell division. However, expression of digestive enzymes is also an important feature of differentiated cells and it would be important to investigate whether such expression could be induced on the RIE-1 cell line by cytokines.

Effects of T Cells and their Products on RIE-1 Cell Growth

I then proceeded to investigate the effects of immune cells and their products on the growth of RIE-1 cells. I first investigated the effects of T cells. There is a substantial amount of circumstantial evidence from clinical studies that T cell mediated immunity is responsible for naturally occurring enteropathy (Mowat, 1984). In addition, a similar pattern of pathology occurs in a number of T cell dependent models in experimental animals, including allograft rejection and intestinal GvHR (Ferguson and Parrott 1972, 1973, Mowat and Ferguson, 1982). Also, activation of T cells in organ cultures of foetal intestine causes crypt hyperplasia, followed by villus atrophy (MacDonald and Spencer, 1988). However, these studies of intact intestine have not identified the mechanisms of action of the T cells. In chapter 2, I looked at the effects of activated T cells on RIE-1 cell cultures. MLN cells were used as a source of T cells and were activated with the T cell-specific mitogen ConA. My results showed that ConA activated MLN cells had a cytostatic effect on the growth of the RIE-1 cells, whereas unactivated MLN cells had little inhibitory effect. As the activated T cells were themselves proliferating in the cultures, it was possible that the cytostatic effect was caused by competition for nutrients, rather than any direct activity against the RIE-1 cells. That this was not the case was demonstrated by the fact that supernatants from ConA activated MLN cells also had a marked cytostatic effect on the RIE-1 cells. Supernatants from unactivated MLN cells had no effect on the growth of RIE-1 cells. Others have shown that supernatants from human T cells activated by anti-CD3 antibody are inhibitory to the HT-29 colonic carcinoma epithelial cell line, whereas supernatants from unactivated T cells had no effect (Deem et al., 1991). However,

unlike my studies, these previous studies showed that T cell supernatants were also cytotoxic to epithelial cells, perhaps reflecting the tumorigenic nature of the HT-29 cells, or the different methods of T cell activation used. Contradictory results have also been reported, where supernatants from both resting or activated human lamina propria mononuclear cells stimulated DNA synthesis in the HT-29 cell line (Lowes et al., 1992a). The reasons for the discrepancy between these earlier studies are unclear and are underlined further by the fact that Deem et al found that IFNγ had a cytostatic effect on HT-29 cells, whereas Lowes et al found that it had no effect. The well known cytostatic effects of IFNγ on many other epithelial cell types suggests that the latter work is incorrect. Another study has shown that supernatants from unstimulated PBMC enhanced the proliferation of keratinocytes, whereas those from stimulated PBMC were inhibitory to growth (Hancock et al., 1988). The latter results were carried out on normal keratinocytes in serum free conditions and, as my experiments were carried out in serum containing medium, any stimulatory effects may have gone undetected. However, the inhibitory effects of activated PBMC on keratinocytes are in agreement with my own.

As IFNγ is a major product of activated T cells and is cytostatic to a wide number of cell types (Trinchieri and Perussia, 1985), I explored whether this mediator might account for the effects of the supernatants from activated MLN cells. I was not able to measure IFNγ directly, but addition of anti-IFNγ antibody reversed the growth inhibitory effects. The effects of the antibody were only partial, but due to a limited supply of antibody, I was unable to determine whether this reflected incomplete blocking of all the IFNγ present or

whether an additional mediator was also involved. However, I was able to show that TNFα did not appear to be important for the effects of my supernatants, as no effect of blocking with anti-TNFα antibody and no bioactive TNFα was present in the supernatants. This contrasts with an earlier report that both IFNγ and TNFα were responsible for the cytotoxic effects of anti-CD3 activated T cells on HT-29 cells (Deem et al., 1991).

This evidence that IFNγ was at least partly responsible for the direct effects of activated MLN on the growth of intestinal epithelial cells was supported by the experiments in chapter 3, which showed that IFNγ itself has a dose dependent inhibitory effect on the growth of both exponentially growing and confluent cultures of RIE-1 cells. IFNγ did not appear to have any cytotoxic effects on the cultures of RIE-1 cells.

These *in vitro* findings are consistent with *in vivo* experiments which suggest a role for IFNγ in enteropathy. Increased levels of IFNγ are produced by lymphoid cells during the proliferative stages of an intestinal GvHR in mice (Garside et al., 1994) and depletion of IFNγ with monoclonal antibody prevents the development of enteropathy in GvHR (Mowat, 1989). Administration of IFNγ also causes keratinocyte hyperplasia in the skin of humans with leprosy (Kaplan et al., 1987). However, it is important to note that these *in vivo* studies suggest that IFNγ has a proliferative role in immunopathology, including enteropathy, whereas my own and other work shows that IFNγ has a profound cytostatic effect on epithelial cells *in vitro* (Symington, 1989, Weetman and Rees, 1988, Campbell et al., 1988). The reason for this discrepancy is unknown, but the results suggest that IFNγ does not act directly on enterocytes *in vivo* and exerts its

effects via other immune cells such as macrophages and NK cells or via mesenchymal cells.

One effect of IFNγ which occurred *in vitro* and was paralleled *in vivo* was its ability to increase the expression of MHC molecules on RIE-1 cells. In conjunction with this, IFNγ also increased the synthesis of total protein by RIE-1 cells. This is consistent with the increased expression of MHC class II molecules which occurs in both crypt and villus epithelial cells in the small intestine during clinical and experimental enteropathies (Arnaud-Battandier et al., 1986, Barclay and Mason, 1982). In addition, treatment of normal mice with IFNγ also leads to *de novo* expression of MHC class II molecules in the crypts of the duodenum (Steiniger et al., 1989). Together, these findings suggest that at least a proportion of crypt cells themselves express receptors for IFNγ. However, whether these are part of the dividing population of cells is unknown. It would be important to examine for the presence of IFNγ receptors on crypt cells *in situ* and on cell lines such as RIE-1.

Activated T cells produce a wide range of cytokines other than IFNγ and TNFα, but due to a lack of availability of blocking antibodies for other cytokines, I was unable to investigate their contributory effects in the MLN supernatants, and so in chapter 3 I looked at the effects of individual T cell derived cytokines on the growth of RIE-1 cells.

IL2, a T cell product which is essential for all T cell functions, had no effect on the growth of either exponentially growing or confluent cultures of RIE-1 cells. This was not surprising as there has been no evidence to suggest that IL2 has trophic effects on epithelial cells and also epithelial cells do not express IL2 receptor.

IL4 containing supernatant stimulated the growth of exponentially growing RIE-1 cells, although due to the lack of a control non-transfected CHO supernatant or an anti-IL4 antibody, it was not possible to prove that this effect was due to IL4 itself. IL4 has been shown to play a role in intestinal GvHR, as enteropathy can be prevented in mice by administering soluble IL4 receptor or an anti-IL4 monoclonal antibody (Mowat, unpublished observations). It can also increase the expression of class II MHC molecules on epithelial cells and it has been suggested that crypt epithelial cells have IL4 receptors (Ritter, M. A. unpublished observations). Thus, it is possible that IL4 does indeed stimulate proliferation of epithelial cells *in vivo* to cause crypt hyperplasia. It would therefore be important to follow up my experiment by examining the effects of purified rat IL4 on RIE-1 cell proliferation. Unfortunately, at present purified or recombinant rat IL4 is not available.

IL3 had no effect on the growth of RIE-1 cells. This is despite the fact that an increase in serum levels and an increase in local production of IL3 occurs in the small intestine in GvHR (Crapper and Schrader, 1986). Also, there is activation and hyperplasia of IL3-dependent mucosal mast cells in enteropathy. My results suggest that IL3 does not play a role in crypt hyperplasia. Interestingly, it has been shown that mucosal mast cells also play no pathogenic role in the enteropathy in GvHR (Newlands et al., 1990).

Thus, these results show that with the possible exception of IL4, none of the T cell derived cytokines tested here had stimulatory effects on the growth of crypt epithelial cells *in vitro*. Again, this does not correlate with *in vivo* studies, where T cells and their cytokines have been shown to be crucial for experimental enteropathy

(Ferguson and MacDonald, 1977, Mowat, 1989). However, addition of purified IL2 or IFNγ to organ cultures of small intestine does not produce any features of enteropathy (MacDonald and Spencer, 1988), adding further support to the idea that if such mediators are important then their effects are indirect.

The Effects of Macrophages and their Products on RIE-1 Cells

Macrophages are important in cell mediated immune responses and are activated during several forms of enteropathy (Ptak et al., 1985, MacDonald et al., 1990). In chapter 2, I investigated the effects of macrophages and their products on the growth of RIE-1 cells. Thioglycollate elicited peritoneal macrophages inhibited the growth of RIE-1 cells and this was enhanced by activating the macrophages further with IFNγ. Thus, IFNγ which itself can have direct effects on the growth of epithelial cells *in vitro* can also have indirect effects via activation of macrophages. It is important to note that thioglycollate itself may have had a stimulatory effect on the macrophages, as the thioglycollate elicited macrophages I used have been shown to express high levels of RNA for TNFα (Sinclair and Bradley. Unpublished observations). Additional stimulation with LPS did not alter the inhibitory effects of either unstimulated or IFNγ stimulated macrophages. As LPS induced TNFα production by these cells and I found in chapter 3 that TNFα inhibited the growth of RIE-1 cells, it is surprising that further stimulation of unactivated or IFNγ stimulated macrophages with LPS did not enhance the growth inhibitory effects of these populations. This may reflect the ability of different combinations of stimuli to produce different forms of macrophage activation.

It was possible that the macrophages were inhibiting growth by phagocytosing RIE-1 cells or competing for nutrients. That this was not the case was shown by the fact that supernatants from the different populations of macrophages generally had similar effects on RIE-1 cell growth to the macrophages themselves. In addition, light microscopy showed no evidence of phagocytosis. These results indicate that soluble products of macrophages were probably responsible for the growth inhibition. One feature of macrophage supernatants which did not correlate with the corresponding population of intact cells was that supernatants from macrophages stimulated with LPS had a biphasic effect on RIE-1 cell growth with an initial stimulatory effect followed by an inhibitory effect at later times in culture. In contrast, LPS stimulated macrophages had an entirely inhibitory effect on growth. The biphasic effect of the supernatant seems likely to be due to the presence of at least two different mediators in the supernatant having opposite effects.

The inhibitory effects of the supernatants were not due to prostaglandin production, as indomethacin did not block the growth inhibition of RIE-1 cells by macrophages. NO is also unlikely to be a major component, as L-NMMA had no effect on the activity of most macrophage populations. However, it did block some of the inhibitory effects of LPS-stimulated macrophages. Thus, there may be some role for NO in this population of macrophages. That NO is important in enteropathy *in vivo* has been shown by the fact that L-NMMA can prevent intestinal pathology in mice undergoing GvHR, although my results suggest that NO does not contribute in a major way to enteropathy by a direct action on crypt epithelial cells (Garside et al., 1992).

In my studies, macrophages stimulated with LPS or LPS and IFNγ produced significant amounts of TNFα and addition of an anti-TNFα polyclonal antibody partially reversed these inhibitory effects. In contrast, unstimulated or IFNγ stimulated macrophages did not produce TNFα. Thus, TNFα is at least in part responsible for the growth inhibition caused by LPS-stimulated macrophages populations. This inhibitory effect was confirmed in chapter 3, where recombinant TNFα had inhibitory effects on the growth of exponentially growing cultures of RIE-1 cells. It is important to note that at very high concentrations, TNFα had a growth stimulatory effect on confluent cultures of RIE-1 cells. Thus, TNFα appears to have different effects on epithelial cell growth depending on the stage of the epithelial cell cycle. This stimulatory effect only occurred at very high concentrations of cytokines and it is possible that *in vivo* epithelial cells are not exposed to such doses of this cytokine and thus TNFα may have predominantly cytostatic effects on dividing epithelial cells *in vivo*. The growth inhibitory effect of TNFα on RIE-1 cells is consistent with its inhibitory effects in a number of other systems (Symington, 1989, Campbell et al., 1988, Weetman and Rees, 1988). In addition, although, I found no microscopic evidence that TNFα was cytotoxic to normal RIE-1 cells, other work in the lab has shown that TNFα kills RIE-1 cells treated with actinomycin (Garside et al., 1993). This is consistent with the idea that during the destructive period of enteropathy, TNFα may be cytotoxic to crypt cells which have been already damaged due to cytokines or other mechanisms. TNFα alone had no effect on protein production or MHC expression by RIE-1 cells (except for a slight increase in MHC class I molecules) but synergised with IFNγ to enhance both class I and II MHC molecules. Thus TNFα

may play a role in increased MHC expression by enterocytes *in vivo*. The growth inhibitory effects of TNFα *in vitro* correlate with *in vivo* studies which show that high levels of TNFα in GvHR are associated with the destructive phase of enteropathy (Garside). Thus, macrophage derived TNFα may be responsible for the sudden decrease in crypt cell proliferation at the onset of villus atrophy and also may damage epithelial cells on the crypt and villus, thus contributing to villus atrophy. This hypothesis is clearly simplistic, as macrophages produce many other mediators some of which are stimulatory and some inhibitory. Thus, I also examined the effects of a number of other macrophage derived cytokines on RIE-1 cells directly.

IL1, is another cytokine product of macrophages (Nathan, 1987, Collart et al., 1986) which has been implicated in enteropathy. Anti-IL1 antibody has been shown to prevent pathology in GvHR and IL1 itself induces enteropathy in normal mice (Mowat and Hutton, 1993). In addition, the levels of IL1 in mucosal biopsies of patients with inflammatory bowel disease are increased compared with control biopsies (Brynskov et al., 1992) and, increases in the number of IL1 secreting cells have been reported in patients with inflammatory bowel disease (Mahida et al., 1989). In my studies, IL1 had no effect on the growth of exponentially growing RIE-1 cells, but it had a significant stimulatory effect on the growth of confluent cultures of RIE-1 cells, even at low doses of cytokine. These findings suggest that IL1 may stimulate resting enterocytes to enter cell division and it could be involved in crypt hyperplasia by stimulating cells which under normal conditions would cease proliferating, to continue dividing. Further support for a growth stimulatory role of IL1 comes

from studies with keratinocytes *in vitro* (Ristow, 1987) and it would be important to investigate this in more depth. Unfortunately, I was unable to do this, as I did not have the reagents to examine the role of IL1 in the activity of macrophage supernatants. However, as IL1 is also a product of activated fibroblasts, it is quite likely that during enteropathy *in vivo*, there will be significant amounts of IL1 present in the intestine and that IL1 mediated interactions between epithelial cells, macrophages and the underlying mesenchyme may be important in the pathology (Huleihel et al., 1990, Le et al., 1987).

IL6, a further cytokine product of macrophages has also been shown to be expressed by epithelial cells and implicated in autocrine regulation of epithelial cell turnover (Shirota et al., 1990). However, it had no effect on the growth of exponentially growing RIE-1 cells. It is important to note that this was human IL6 and it is possible that it did not cross react with the rat cytokine. Due to a limited supply of cytokine I was unable to test the effect of IL6 on confluent cultures of RIE-1 cells.

IFNα/β also had no effect on RIE-1 cell growth, despite the fact that it can produce intestinal pathology similar to that seen in enteropathy when injected into normal mice (Garside et al., 1991). However, anti-IFNα/β antibody has no real effect on GvHR in mice and thus may not play a primary causal role in enteropathy, but may have secondary effects via activation of NK cells (Garside, unpublished).

TGFβ is a product of activated macrophages and T cells and, although in chapter 1 I showed it to be growth inhibitory to exponentially growing RIE-1 cells, it had no effect on confluent cultures. Thus, TGFβ appears to act on cells which are actively dividing. Together with studies showing that the expression of TGFβ by intestinal epithelial

cells shows an increasing gradient from crypt to villus, my studies support the hypothesis that TGFβ is an autocrine inhibitory mediator of epithelial cell turnover (Barnard et al., 1989). Production of TGFβ by epithelial cells or activated T cells or macrophages may be the signal which removes crypt cells from the cell cycle.

The Cytotoxic Effects of NK cells on RIE-1 cells

Another immune cell of potential importance in enteropathy is the NK cell. Intestinal and systemic NK activity is enhanced in mice undergoing a GvHR and this activity develops in parallel with the evolution of other proliferative features of intestinal GvHR (Borland et al., 1983, Mowat et al 83, Mowat et al., 1985). Depletion of NK cells with anti-asialo GM1 antibody *in vivo* inhibits systemic GvHR and abolishes intestinal pathology (Mowat and Felstein, 1987, Charley et al., 1983). NK cells are particularly active against dividing cells *in vitro* and I was therefore interested to use RIE-1 cells to examine whether NK cells would be cytotoxic to dividing epithelial cells. However, my experiments showed that NK cells were not cytotoxic to RIE-1 cells in a chromium release assay. Although this suggests that NK cells do not have a cytotoxic role in enteropathy, it is important to note that activated NK cells could still contribute to enteropathy by releasing inflammatory cytokines (Kasahara et al., 1983, Handa et al., 1983).

The Effects of Cytokines on Confluent versus Growing Populations of RIE-1 Cells

One important aspect of the RIE-1 system was that the effects of some cytokines depended on the stage of growth. In many of my

experiments, I used RIE-1 cells which were in the exponential phase of growth, corresponding to dividing crypt cells *in vivo*. However, I also examined the effects of cytokines on confluent cultures, where most of the cells are not proliferating actively. I was interested in this because not all crypt cells are dividing *in vivo*. Although the stem cells proliferate constantly, adjacent crypt cells divide asynchronously, with only one of the two daughter cells dividing again and shortly thereafter, the crypt cells cease to divide altogether. It is possible that these non-dividing cells can be recruited back into the cell cycle, by cytokines such as IL1 which I showed to have a growth stimulatory effect on confluent cultures of RIE-1 cells, and contribute to crypt hyperplasia. Thus, these cells are better represented *in vitro* by confluent cultures of cells rather than exponentially dividing populations of cells.

Together, these results show that T cell supernatants, macrophage supernatants and individual cytokines can have direct effects on the growth of epithelial cells *in vitro*. Supernatants from activated T cells and macrophages were mainly inhibitory to RIE-1 growth. IFNγ was the only T cell product with a reproducible effect on growth, whereas a number of macrophage products had marked effects on RIE-1 growth. With the exception of IL1, most of these effects were cytostatic, although unphysiological doses of TNFα also had growth stimulatory effects on confluent cultures of RIE-1 cells. The generally cytostatic effect of the cytokines contrasts with the hypothesis that cytokines cause crypt hyperplasia during enteropathy via a direct effect on crypt epithelial cells. Although it is possible that the only cytokine effective on epithelial cells *in vivo* is IL1, or that other unknown cytokines have stimulatory effects, the consistently

inhibitory activity of T cell and macrophage products *in vitro*, make this seem unlikely. However, the cytostatic effects caused by many of the cytokines tested may be important at the transition stage, when crypt cell turnover decreases and the destructive phase of enteropathy begins (Mowat and Felstein, 1990).

Co-culture of RIE-1 Cells with Fibroblasts

In view of the fact that it was difficult to reproduce increased proliferation *in vitro* and because the mucosa is very complex *in vivo*, I went on to see if adding another component might make the model more representative of the situation *in situ*. It has been shown that the mesenchyme plays an important role in the control of differentiation in the developing gut and there are close associations between epithelial cells and fibroblasts in the foetal gut (Kedinger et al., 1988, Marsh and Trier, 1974). I therefore attempted to set up co-cultures of RIE-1 cells with mesenchymal cells *in vitro*, using the mouse fibroblast L929 cell line as a source of mesenchymal cells. I had anticipated that the RIE-1 cells would grow as a monolayer on the surface of a confluent layer of L929 cells, but many of the RIE-1 cells appeared to push aside the L929 cells. Although the remaining RIE-1 cells did appear to be influenced by the fibroblasts, growing in multilayered structures and being much less flattened than usual, there were few, if any, other signs of differentiation in this system. Together with the fact that there seemed to be death of the fibroblasts in the co-cultures, I concluded that this system would not be useful to study the effects of cytokines on co-cultures of epithelial cells and fibroblasts. The reasons for these difficulties are unclear, especially as IEC-17 cells, a similar epithelial cell line, have been co-

cultured successfully with intestinal mesenchyme (Kedinger et al., 1986). However, other workers have shown that while a differentiated epithelial cell line will grow as a monolayer in co-culture, an undifferentiated cell line will not and produces similar results to my co-cultures (Bouziges et al., 1991). An additional possibility is that the fibroblasts I used were not of rat origin, and although previous work has suggested that species specificity is not necessary in co-cultures of endoderm and mesenchyme (Haffen et al., 1983), organ specific receptors for attachment and other inductive properties might be required. It might also have been preferable to have used mesenchymal cells derived from the gut, particularly if it had been possible to obtain a primary culture of gut fibroblasts rather than a cell line. Finally, the fact that the RIE-1 cells have been adapted to growth *in vitro* may mean that they are unable to grow and differentiate on a mesenchymal layer. In future work, it would be important to attempt to establish such a co-culture system using some of the modifications discussed above.

RIE-1 Cells as a Model for Investigating Enteropathy

Although altered cell turnover is the prominent feature of epithelial cell pathology in enteropathy, this is accompanied by changes in function which affect the differentiation of disaccharidases and class II MHC molecules, as well as permeability to ions (Lund et al., 1986, Barclay and Mason, 1982). Although I found that IFNγ increased protein synthesis and MHC expression by RIE-1 cells, there were no ultrastructural changes in the cells which might suggest differentiation. In addition, no other cytokine affected protein synthesis by RIE-1 cells, or induced any features of differentiation.

This included TGFβ, a mediator thought to play an important role in differentiation. RIE-1 cells were also unable to form functional tight junctions, as assessed by transmembrane potential differences, even after long term culture at confluence under conditions which promoted tight junction formation by Caco-2 cells. Together with failure to grow in co-cultures with fibroblasts, these results suggest that RIE-1 cells cannot differentiate. Thus, their use as an *in vitro* model may be limited to the study of factors which modulate enterocyte proliferation. Nevertheless, it would be worth further attempts to induce these cells to differentiate by culturing them in glucose free medium which has been successful in other systems (Pinto et al., 1982). In addition, as TGFβ has been shown to increase expession of sucrase by another epithelial cell line (Kurokowa et al., 1987), it would be important to examine if any of the cytokines or T cell and macrophage supernatants could alter the expression of enzymes by RIE-1 cells.

Primary Cultures of Intestinal Epithelial Cells

The results of my studies with RIE-1 cells indicate that they have limitations as an *in vitro* model of epithelial cells. Therefore, I thought it important to try and develop a model of primary epithelial cell growth *in vitro*. This would have several potential advantages including the fact that the cells in primary culture are taken directly from *in vivo* and are thus likely to retain more features of enterocytes *in vivo* than cultured cell lines. Although numerous previous studies had been unable to propagate cultures of intestinal epithelial cells in the absence of contaminating mesenchymal cells (Raul et al., 1978, Kondo et al., 1984), at the time I began my study, it appeared that

Evans and colleagues had developed a novel method for isolating and growing rat small intestinal epithelial cell cultures *in vitro* (Evans et al., 1992). I therefore decided to try and establish this system as a means of examining the effects of cytokines on fresh, dividing enterocytes.

My initial studies showed that intact epithelial fragments could be isolated and maintained in culture over a period of at least 10 days. During this time, the cells proliferated steadily and, by the end of the period, almost completely filled the well. Early in culture, light microscopy demonstrated small colonies of tightly packed polygonal cells, which had the appearance of epithelial cells. With time, these colonies grew and joined up to form sheets of cells. Although this was preliminary evidence that this model would provide a means of studying dividing epithelial cells *in vitro*, other areas of the cultures appeared to consist of non-epithelial cells, many of which had the appearance of fibroblasts. These findings were confirmed by EM, which showed the presence of epithelial cells, characterised by polarisation, tight junctions, desmosomes and microvilli, but also the presence of other flattened cells with no features of epithelial cells. Again, these appeared to be fibroblasts. Cytokeratin staining was also used to identify epithelial cells in the cultures and, initially, almost all the cells stained positive in the culture. However, with time, the proportion of positive cells decreased, confirming the presence of contaminating cell types.

Despite investigating several modifications of the culture conditions aimed at reducing the growth of non-epithelial cells, such as lowering the concentration of serum and addition of heparin, it was impossible to avoid the contamination by other types. These findings indicating

that this method would not provide pure cultures of epithelial cells *in vitro* have recently been confirmed by Evans and colleagues (Evans et al., 1992). Despite the fact that a mixed culture of this type would be a complex model for investigating the effects of cytokines on epithelial cells, I decided to examine effects of a number of mediators on the primary cultures I was able to establish. This would allow comparison with the experiments using the RIE-1 cell line, and perhaps give some indication of epithelial/mesenchymal interactions. However, none of the cytokines or supernatants I used had any effect on the growth or appearance of the primary cultures. Addition of different concentrations of EGF to the cultures also had no effect on growth, suggesting that this system was not appropriate for assessing the effects of growth factors in general. This may reflect the presence other growth stimulators such as insulin and FCS, which were components of the culture medium needed for establishing the cultures. It is also possible that the cytokines were exerting opposing effects on the different cell types present in the culture. These results contrasted markedly with the growth modulatory effects I observed using the RIE-1 cell line and highlight the need for a suitable model for obtaining primary cultures of small intestinal epithelial cells *in vitro*. One possible means of doing this may be that devised by Haffen, in which foetal rat endoderm, consisting of pure epithelial cells is isolated and cultured on a layer of mesenchyme until confluence is reached (Haffen et al., 1981). This produces a monolayer of epithelial cells with the characteristics of mature enterocytes *in vivo*. If this method could be adapted to employ irradiated mesenchyme, it could offer a suitable model in which

quantitative assays of growth could be used to investigate the effects of cytokines on intestinal epithelial cells.

CONCLUSIONS

The results in this thesis have highlighted the difficulties in studying the interaction between the immune system and the intestinal epithelium *in vitro*. My work shows that the RIE-1 cell line may provide a useful *in vitro* model of crypt epithelial cells, but its use is limited by its apparent inability to differentiate. My attempts to set up a primary model of pure intestinal epithelial cells were unsuccessful and to date no such model is available.

Using the RIE-1 cell line as a model, I found that soluble products of T cells and macrophages could have direct effects on the growth of dividing intestinal crypt cells *in vitro*. With the exception of a factor produced from LPS stimulated macrophages which was growth stimulatory, all the supernatants I studied inhibited the growth of RIE-1 cells. Most purified macrophage or T cell-derived cytokines also had a cytostatic effect on RIE-1 cells, although IL1 and TNFα stimulated the proliferation of confluent RIE-1 cells. These findings were against the hypothesis that cytokines produced during a cell mediated immune response cause crypt hyperplasia due to a direct growth stimulatory effect on dividing crypt stem cells. Nevertheless, it remains possible that cytokines such as IL1 might contribute to hyperplasia by inducing resting crypt cells to enter cell division.

The principal defect of the RIE-1 cells as a model system was their inability to behave like enterocytes *in vivo* by differentiating or growing in association with a fibroblast monolayer. Although IFNγ and TNFα did induce the expression of MHC molecules, which may be

markers of altered differentiation *in vivo*, no other features of differentiation could be induced in RIE-1 cells using cytokines or fibroblasts.

Together with the fact that primary cultures were only successful if contaminating mesenchyme was present, I consider that if cytokines play a pathogenic role in enteropathy, this reflects an action via the mesenchymal layer of cells associated with the epithelium *in vivo*. Further work should concentrate on establishing co-culture models or primary culture systems containing defined populations of cell types in which these interactions can be studied directly.

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