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Antigen uptake and trafficking in human intestinal epithelial cell lines

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by

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ABSTRACT

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In addition to its physiological function as an absorptive cell, the intestinal epithelial cell plays an active role in regulating gut mucosal immune responses. Several laboratories have demonstrated the antigen presenting cell properties of intestinal epithelial cells (IEC) in vitro. In the presence of soluble antigens, the IEC from rats, mice or humans can induce MHC-restricted antigen-specific T cell proliferation. Most interestingly, the T cell population activated by the IEC are predominately $CD8^+$ suppressor T cells. The exact mechanisms of $CD8^+$ suppressor T cell activation by IEC are still not fully understood. One plausible contributing factor may be that antigens are handled differently by the IEC when compared with conventional antigen presenting cells (APC) in systemic immunity.

To define the unique properties of IEC, the pathways that IEC employ to handle antigens were compared and contrasted with those of conventional APC. Utilizing fluorescence, electron, and laser scanning confocal microscopy, we showed that the kinetics of antigen uptake by IEC lines was slower when compared with that of monocytes. Fluorescein-conjugated tetanus toxoid was internalized only after 30 min incubation at 37°C compared with 5 min for monocytes. However, similar to monocytes, exogenous soluble protein antigens were taken up by fluid phase endocytosis and followed a classical class II pathway from early endosomes to late endosomes and subsequently into lysosomal compartments. The observation that tetanus toxoid (gold-labeled or fluorescein-labeled) resided in endosomal and lysosomal compartments may have significance with respect to the site(s) of antigen processing in IEC since MHC class II molecules have been demonstrated in these compartments. In the polarized intestinal epithelial cell line, Caco-2, antigen applied apically did not traffic to the basal cytoplasm but remained in apical lysosomal compartments, whereas antigen applied basally transcytosed to the apical cytoplasm. We also analyzed factors (antigen size, immune complexes, and prophagocytic cytokines) which might alter antigen uptake by IEC. Our data showed that, similar to monocytes, the size of antigen (e.g. OVA, KLH) played no role in antigen uptake. However, in contrast to phagocytic monocytes, IEC failed to take up insoluble antigens.

Prophagocytic cytokines (IFN-γ or GM-CSF) and soluble immune complexes did not enhance antigen uptake while they did in monocytes. Taken together, our findings show that antigen uptake by IEC is a tightly regulated and stable process. While IEC are less efficient APC when compared with monocytes, the manner whereby antigen is handled may play an important role in maintaining a suppressed immunologic tone in the gastrointestinal tract.

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ABBREVIATIONS

APC	antigen presenting cells
DAMP	3-(2,4-dinitroanilino)-3'-amino-N-methyldipropylamine
DNP	dinitrophenol
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
GALT	gut-associated lymphoid tissue
GI	gastrointestinal
GM-CSF	granulocyte-macrophage colony stimulating factor
IBD	inflammatory bowel disease
IEC	intestinal epithelial cells
IEL	intraepithelial lymphocytes
IFN-γ	gamma-interferon
Ig	immunoglobulin
KLH	keyhole limpet hemocyanin
LMP	low molecular mass complex
LP	lamina propria
LPL	lamina propria lymphocytes
M6PR	mannose-6-phosphate receptor
MHC	major histocompatibility complex
OVA	ovalbumin
pIgR	polymeric immunoglobulin receptor
PMN	polymorphonuclear neutrophils

PP	Peyer's patches
sIgA	secretory IgA
TCA	trichloroacetic acid
TT	tetanus toxoid

INTRODUCTION

In order to maintain homeostasis in an environment that is bombarded by a myriad of antigens, the manner by which the mucosal immune system handles antigens must be distinct from that of the systemic immune system. This is evident by the types of antigen presenting cells (APC) that exist in the gut. In addition to the conventional APC in the Peyer's patches (PP) and lamina propria (LP), the intestinal epithelial cells (IEC) appear to play a role in regulating mucosal immune responses. Therefore, one might postulate that focusing antigen on one cell versus another may alter the type of immune response generated. The predominant immune response in the gut is a non-response. The paucity of effective oral vaccines attests to this concept. More specifically the phenomenon of oral tolerance, predominantly induced by soluble antigens, is thought to be effected by the generation of suppressor T cells. The mechanism of antigen trafficking in the gut might therefore dictate whether active immunity or tolerance will result. This may either be reflective of the type of cell involved as the APC (IEC vs. PP macrophages or LP macrophages) or the route that antigen takes through the cell. Our laboratory has previously shown that soluble protein antigens processed by IEC induce a $CD8^+$ suppressor T cell response. In contrast, bacteria and viruses that bind to the IEC may traffic differently within the IEC resulting in a non-tolerizing response. Lastly, antigens which bind to receptors on the IEC may follow a different path resulting in yet another type of response. In

this section, the roles of intestinal epithelial cells, the pathways of antigen entry as well as trafficking are discussed to give a general background for this thesis.

PART I: ROLE OF INTESTINAL EPITHELIAL CELLS IN MUCOSAL IMMUNE RESPONSES

GUT-ASSOCIATED LYMPHOID TISSUE

Although the gastrointestinal (GI) tract encounters a wide variety of antigens derived from different sources, immune responses are tightly regulated and continuously kept in check by gut-associated lymphoid tissue (GALT) to ensure that overwhelming inflammation does not occur. GALT, composed of Peyer's patches, mesenteric lymph nodes, lamina propria lymphocytes (LPL), and intraepithelial lymphocytes (IEL), is the largest mucosa-associated lymphoid tissue in the body. GALT is distinct from the systemic immune system in many ways. Secretory IgA (sIgA) is the predominant antibody in the mucosa in contrast to IgG systemically (Heremans, 1974). In addition, the T cell subpopulations found in gut mucosal tissues are unique. Most of the LPL are of memory cell ($CD45RO^+$) phenotype (Schieferdecker *et al.*, 1990) and the majority of the IEL are $CD8^+$ (Cerf-Bensussan *et al.*, 1983). GALT does not work alone to maintain homeostasis in the gut. The intestinal epithelium is an intimate partner of GALT and plays an active role in immunosurveillance of the GI tract.

MUCOSAL BARRIER

One of the major ways to maintain homeostasis in the GI tract is to control the entry of antigens through the mucosa so that immunocompetent cells underlying the intestinal epithelium do not become activated and induce inflammation. Most luminal antigens are either cleared by peristalsis or digested into smaller components. However, some macromolecules and pathogens manage to escape digestion and peristalsis. To impede the entry of these pathogens and macromolecules, the intestinal epithelium also secretes a viscous mucus coat to inhibit the adherence of bacteria to their surface and to trap macromolecules. In addition to these nonspecific mucosal barriers, GALT produces antigen-specific sIgA which excludes the entry of antigens (Challacombe and Tomasi, 1980). Dimeric sIgA secreted by plasma cells in the lamina propria binds to the secretory component expressed on the basolateral surface of IEC (Brown *et al.*, 1977). Together, sIgA and the secretory component are transported to the apical surface and released into the lumen. The binding of sIgA to antigen forms immune complexes, which not only prevent the entry of antigens but render the antigen more vulnerable to proteolysis (Pang *et al.*, 1981). Lastly, the intestinal epithelium, by virtue of the presence of tight junctions between the cells, serves as a physical barrier to limit the entry of antigen (Gumbiner, 1987). Controlled antigen entry is essential for homeostasis in the gut because any breakdown in mucosal barrier function allows for the transit of macromolecules and pathogens across the mucosa, resulting in certain gastrointestinal diseases (Sanderson and Walker, 1993).

ORAL TOLERANCE

Unique to the intestinal immune system is the fact that some orally administered antigens induce antigen-specific oral tolerance (immunologic nonresponsiveness of systemic immune response). Induction of antigen-specific immune suppression by orally fed antigens was observed over a hundred years ago by Dakin (1829) who reported that South American Indians avoided contact hypersensitivity to urushiol by eating the leaves of poison ivy. Yet, the definitive report of oral tolerance was first described by Wells (1911) in which he showed that prior antigen feeding could prevent systemic anaphylaxis in guinea pigs. While the exact mechanisms responsible for the induction of oral tolerance are not fully understood, $CD8^+$ suppressor T cells have been implicated as playing an important role (Ngan and Kind, 1978; Mattingly and Waksman, 1978). Several studies have shown that adoptive transfer of splenocytes, especially $CD8^+$ T cells, from orally tolerized animals to naive animals can transfer systemic tolerance to specific antigens (Richman *et al.*, 1989). However, the mechanism whereby $CD8^+$ suppressor T cell activation occurs in oral tolerance has not been fully elucidated. In addition to the suppressor T cell model, Ferguson *et al.* (1988) have proposed another mechanism for the induction of oral tolerance. They suggest that oral administration of antigens results in the generation of tolerogenic molecules from "gut processing." In their studies, such tolerogenic molecules derived from ovalbumin were recovered in the serum of ovalbumin-

fed mice. Further studies demonstrated that these tolerogenic molecules were capable of inducing tolerance when transferred intraperitoneally to naive mice (Strobel *et al.*, 1983). Other models proposed have included the production of anti-idiotypic antibodies (Jackson *et al.*, 1981), suppressive immune complexes (Andre *et al.*, 1975), and the anergy of T cells (Melmed and Friedman, 1993). However, these models have not been confirmed by other groups.

One interesting observation made by several laboratories is that the IEC are capable of presenting soluble antigens to stimulate major histocompatibility complex (MHC) restricted antigen primed T cells (Bland and Warren, 1986a; Mayer and Shlien, 1987; Kaiserlian *et al.*, 1989). However, in contrast to conventional APC, T cells which are activated by the IEC are predominantly $CD8^+$ suppressor T cells (Bland and Warren, 1986b; Mayer and Shlien, 1987). These findings raise the question as to whether the way(s) in which antigens are handled by the IEC might be responsible for T suppressor cell activation, which in turn might account for the phenomenon of oral tolerance and controlled inflammation in the gut.

MHC CLASS II EXPRESSION IN INTESTINAL EPITHELIAL CELLS

The presence of MHC class II molecules on the intestinal epithelium was first observed in guinea pigs (Wiman *et al.*, 1978). Thereafter, similar observations were made in rats (Mason *et al.*, 1981), mice (Parr and McKenzie, 1979), and humans (Scott *et al.*, 1980). Only mature absorptive epithelial cells have been found to express MHC

class II molecules (Bland, 1988). The expression of MHC class II molecules is greater in the small intestine than in the large intestine, and greater in villus cells than in crypt cells (Bland and Kambarage, 1991). Whereas Mayer *et al.* (1991) demonstrated that MHC class II molecules were expressed predominantly on the apical surface in enterocytes by immunohistochemistry, Mayrhofer and Spargo (1989), utilizing electron microscopy, found that most MHC class II molecules were located on the basolateral membranes, in multivesicular bodies (late endosomes) in the apical cytoplasm, and lysosomes. The localization of MHC class II molecules in endosomal and lysosomal compartments, which are hypothetical antigen processing and coupling sites in conventional APC (Berzofsky *et al.*, 1988; Harding *et al.*, 1990; Peters *et al.*, 1991), strongly suggests that MHC class II molecules have a probable role in antigen presentation by IEC. MHC class II molecules are constitutively expressed on IEC and can be upregulated by gamma-interferon (IFN-γ; Salomon *et al.*, 1991). Interestingly, HLA-DQ molecules are not expressed on IEC despite the presence of HLA-DQ mRNA (Mayer *et al.*, 1991). Even in states of inflammation, HLA-DQ expression is not upregulated like HLA-DP and HLA-DR. The significance of this observation is not fully understood. IEC from inflammatory disease states, such as inflammatory bowel disease (IBD), express higher levels of MHC class II molecules which are attributed to increased IFN-γ secretion by the LPL and IEL in the local environment (Salomon *et al.*, 1991). In addition, Mayer and Eisenhardt (1990) noted that IEC from patients with IBD failed to activate $CD8^+$ suppressor T cells. The lack of induction of suppressor T cells apparently is not related

to increased MHC class II expression because the IEC from patients with diverticulitis or ischemic colitis, where MHC class II expression was comparable to that in IBD, still stimulate $CD8^+$ T suppressor cells (Mayer and Eisenhardt, 1990). Lastly, since MHC class II molecules are expressed on the intestinal absorptive epithelial cells and protein re-absorbing proximal tubules in the kidney (Mayrhofer and Schon-Hegard, 1983), Bland (1988) has proposed that, in addition to antigen presentation, MHC class II molecules may act as peptide receptors, which selectively bind to immunogenic peptides generated by luminal preprocessing, and shuttle them from the apical to basolateral side in IEC during antigen handling.

ANTIGEN PRESENTATION BY INTESTINAL EPITHELIAL CELLS

The presence of MHC class II molecules on the cell surface and in endocytic compartments does not necessarily guarantee that IEC can function as APC. The antigen presenting function of IEC was first explored by Bland and Warren (1986a) using a rat model system. They showed that rat enterocytes could process ovalbumin and present ovalbumin peptides to selectively activate antigen-specific $CD8^+$ suppressor T cells (Bland and Warren, 1986b). As in conventional APC, antigen presentation in enterocytes could be blocked by lysosomotropic agents, chloroquine and ammonium chloride, as well as monensin (Na^+-H^+ ionophore that disrupts the acidic pH in endosomes). Leupeptin (thiol protease inhibitor), on the other hand, had no effect on antigen presentation, suggesting that

different enzymes are involved in the processing of ovalbumin by IEC compared to conventional APC (Bland and Whiting, 1989). In addition, when comparing lysates from enterocytes vs. splenocytes after antigen pulsing, minimal processing of ovalbumin was observed in intestinal epithelial cell lysates. Therefore, it appears that IEC are less effective in antigen processing than conventional APC. Santos *et al.* (1990) also reported that rat IEC could process and present myelin basic protein to primed T cells, but only in the presence of indomethacin, a cyclooxygenase inhibitor, suggesting that IEC secreted prostaglandins, which had been shown to induce nonspecific suppressor T cells (Fischer *et al.*, 1981) or be inhibitory in their own right. However, since such inhibition would be a local phenomenon and antigen-nonspecific, prostaglandin is not likely to be a mediator of antigen-specific oral tolerance. It is more likely that it plays a role in the general suppressed tone of the gut. Antigen processing and presenting functions were also observed in human IEC by our laboratory in a tetanus toxoid specific system (Mayer and Shlien, 1987). This T cell response to tetanus toxoid was blocked by fixing IEC with paraformaldehyde indicating that processing is essential for antigen presentation. One finding that distinguishes the human intestinal epithelial cell system from Bland's rat intestinal epithelial cell system is that the $CD8^+$ suppressor T cells that were activated by human IEC were antigen-nonspecific. IEC from mice have also been shown to be capable of presenting keyhole limpet hemocyanin (KLH) to a $CD4^+$ antigen-specific T cell hybridoma (Kaiserlian *et al.*, 1989). Since a $CD4^+$ T cell hybridoma line was used in this system, T suppressor cell

activation was not noted. Nevertheless, this observation has definitely lent support to the postulate that IEC, aside from their function as absorptive cells, have a novel role -- antigen presentation -- in the intestinal immune system.

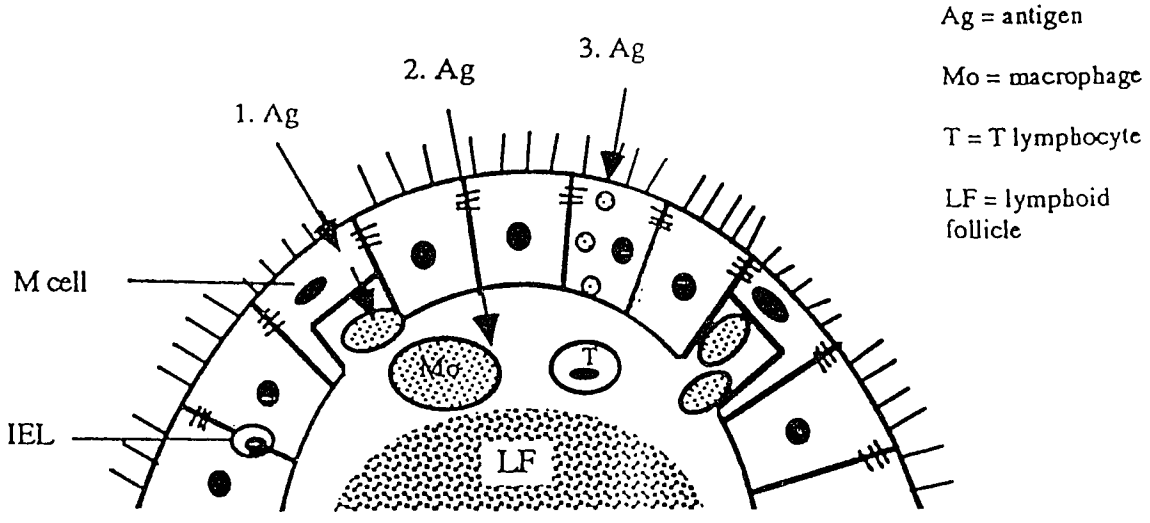
Part II: ANTIGEN UPTAKE BY INTESTINAL EPITHELIAL CELLS

ANTIGEN ENTRY

Immunosurveillance in the GI tract involves antigen uptake and sampling by the IEC. Thus, it is important to understand how luminal antigens gain access to the immunocompetent T cells lying between the IEC and in the lamina propria. Before discussing the mechanisms of antigen entry, it is helpful to understand the physical and physiological relationships of the intestinal epithelium with LPL and IEL. As opposed to LPL, which consist of 60% T cells and 40% B cells, IEL are predominantly T cells (Selby *et al.*, 1981). Further phenotypic analysis of the T cell populations of IEL and LPL reveals that the ratio of $CD8^+$ to $CD4^+$ T cells in IEL is 9:1 compared to 1:2 in LPL (Selby *et al.*, 1983). In addition, several studies have suggested that the intestinal epithelium may be actively involved in extra-thymic differentiation of T cells since IEL are still present in mice with severe combined immunodeficiency disease, where thymic-induced differentiation is absent. In these mice, 20-50% of their IEL are still $CD8^+$ with CD8 molecules made exclusively of CD8 α-α homodimers as opposed to CD8 molecules on thymic-derived $CD8^+$ T cells which are CD8 α-β heterodimers (Croitoru *et al.*, 1990; Guy-Grand *et al.*, 1991). Another feature of IEL, which exist juxtaposed to the intestinal epithelium, is that they are difficult to activate by conventional stimuli (e.g. anti-CD3 monoclonal antibodies, concanavalin A, phytohemagglutinin, superantigens etc.) although they appear to be constitutively activated in vivo ($CD45RO^+$, $IL\text{-}2R^+$, $p56^{lck}$ activated). In humans, a cocktail of IL-2

and anti-CD2 monoclonal antibodies is one of a small number of stimuli that can activate IEL in vitro (Ebert *et al.*, 1986). These findings raise questions as to whether the interaction of IEL with the intestinal epithelium results in the unresponsiveness described. Although LPL do not contact the intestinal epithelium with such intimacy, IEC have been shown to send pseudopod-like processes through the porous basement membrane (Mathan *et al.*, 1972; McClugage and Low, 1984). Thus, the IEC are still able to make direct contact with LPL.

Since the key to drive any immune response is the antigen, it is essential for antigens to cross the intestinal epithelium and interact with the immunocompetent lymphocytes underlying the epithelium. Figure 1 illustrates three possible routes by which luminal antigens can traverse the epithelium before meeting LPL and IEL: 1) M (membranous epithelial) cells; 2) paracellular route; 3) transcellular route.



M CELLS

M cells are specialized intestinal epithelial cells that are interspersed among the columnar absorptive epithelial cells in the dome epithelium overlying lymphoid follicles, especially the Peyer's patches, in the GI tract (Bockman and Cooper, 1973; Owen and Jones, 1974). Morphologically, they have a thinner cytoplasm as well as shorter and fewer microvilli which distinguish them from columnar absorptive epithelial cells. Macrophages and lymphocytes are often found within the intercellular pockets of M cells, raising the possibility that M cells might be responsible for antigen handling in the gut (Owen and Ermak, 1990). Previous studies, however, have shown that M cells do not possess any lysosomes (Owen *et al.*, 1986a), do not process luminal antigens (Owen, 1977), and do not express MHC class II molecules (Bjerke and Brandtzaeg, 1988). In other words, these data support the fact that M cells are incapable of processing and presenting antigens to underlying lymphocytes but rather may serve as a conduit for antigen passage to macrophages and subsequently into the Peyer's patches. Recently, Allan *et al.* (1993) have reported that acidic endosomal-lysosomal compartments and MHC class II molecules are present in rat intestinal M cells and speculated that M cells may have the potential to be APC. However, given that they constitute only 5%-10% of the dome epithelium in humans (Brandtzaeg and Bjerke, 1989), it is quite unlikely that they are the major APC in the intestinal immune system. It is well documented that M cells provide entry for particular micro-organisms, such as *Vibrio cholerae* (Owen *et al.*, 1986b) and reovirus (Wolf *et al.*, 1981). These microorganisms and

many macromolecules, like ferritin, preferentially adhere to M cells where they traverse the mucosal barrier. Interestingly, the molecules or organisms that traffic via the M cells have not been reported to induce oral tolerance. In fact, this may explain why the live attentuated poliovirus vaccine is effective in inducing systemic immune responses since poliovirus has been demonstrated to have a high affinity for M cells (Sicinski *et al.*, 1990). M cells transport the virus to underlying macrophages for conventional antigen presentation to activate B and T lymphocytes in the Peyer's patches. Primed T and B cells in the Peyer's patches then travel to the systemic circulation. In addition, sIgA and IgG have been shown to bind selectively to M cells and are capable of being transported transepithelially. The transport of IgA-antigen complexes might help augment the existing secretory immune responses (Weltzin *et al.*, 1989). Furthermore, it has been reported that increased transport of hydroxyapatite-conjugated antigen (e.g. ferritin) by M cells is correlated with increased immunogenicity of the antigen and IgA responses (Amerongen *et al.*, 1991; Amerongen *et al.*, 1992). Another interesting observation involves the preferential binding of gold-conjugated cholera toxin to M cells, possibly due to the fact that the GM1 ganglioside, cholera toxin receptor, on M cells was more accessible than that on the IEC (Neutra and Kraehenbuhl, 1994). The mucosal adjuvant activity of cholera toxin is well established, yet the mechanisms for its immunogenicity are not fully elucidated (Elson and Ealding, 1984). The finding that cholera toxin binds to M cells might, in part, help account for its immunogenicity. However, no direct evidence exists for this assumption. Thus, the data to date

support the induction of active immune responses other than suppression for antigens which traffic through the M cells.

PARACELLULAR TRANSPORT

The intestinal epithelium exists as a polarized sheet of cells joined together by the tight junctions (Rodriques-Boulan and Nelson, 1989). In addition to maintaining polarity and forming a physical barrier, the tight junctions have been described by Madara *et al.* (1992) to be the "rate limiting barrier" in intestinal transport. They have shown that in a glucose-induced state, small quantities of peptide fragments (500-1500 dalton) can pass through the tight junctions and appear in the portal blood (Atissok and Madara, 1986; Webb, 1986). Tight junctions, however, can be broken down by *Clostridium difficile* toxin A (Hecht *et al.*, 1988) and IFN-γ (Madara and Staffork, 1989). In several disease states where inflammation is present, such as Crohn's disease, increased production of IFN-γ by activated T cells might enhance paracellular transport of macromolecules. In addition, Nash *et al.* (1987) showed that transmigration of polymorphonuclear neutrophils (PMN) via the tight junctions resulted in barrier defects. Although IL-8, a potent PMN chemoattractant, has been demonstrated to be secreted by IEC upon the entry of certain enterobacteria (Eckmann *et al.*, 1993), McCormick *et al.* (1993) have reported that IL-8 alone is not sufficient to cause PMN transmigration. Another unidentified transcellular chemoattractant may also be required. Furthermore, Lycke *et al.* (1991) proposed that the adjuvant activity of cholera toxin might be associated with

enhanced intestinal permeability induced by cholera toxin, thus resulting in increased entry of luminal antigens, possibly via the paracellular route to the lamina propria. Collectively, disruption of tight junctions results in increased antigen entry through the intestinal epithelium to the underlying lymphoid tissues, which may in turn add to ongoing inflammation by inducing active mucosal immune responses.

TRANSCELLULAR TRANSPORT

Most orally administered antigens are broken down in the stomach and small intestine. Brush border associated aminopeptidases in IEC allow further breakdown of luminal proteins and peptides into amino acids before absorption. IEC are known to be capable of absorbing di-peptides and tri-peptides (Bloch *et al.*, 1988). A small percentage of dietary proteins, however, escape preprocessing and enter the systemic circulation (Hersby *et al.*, 1985). Bockman and Winborn (1966) reported that intact macromolecules like ferritin were taken up by fluid phase pinocytosis in IEC and transported to the lamina propria and capillaries. Similar observations were made by Walker *et al.* (1972) who studied the transcellular transport of horseradish peroxidase in rat IEC. In suckling rats, growth factors (e.g. epidermal growth factor, nerve growth factor etc.) taken up by receptor-mediated endocytosis have also been shown to traverse the IEC to the basolateral cell surfaces and lamina propria (Blay and Brown, 1985; Gonnella, *et al.*, 1987). In addition, Abrahamson and Rodewald (1981) reported that in newborn rats, IgG from maternal

milk could bind to Fc receptors on the IEC and be internalized by receptor-mediated endocytosis into the apical endosomes where sorting occurred. IgG-receptor complexes were transcytosed to the lateral side, whereas non-receptor-bound proteins were delivered to the lysosomes. Using the Caco-2 cell line (malignant small bowel-like), Heyman *et al.* (1990) demonstrated that less than 10% of apically applied horseradish peroxidase was transported in an intact form after traversing the IEC. The majority of horseradish peroxidase was degraded by lysosomes. Based on these findings, it appears that most of the soluble proteins internalized by nonspecific endocytosis at the apical surface are directed to lysosomes in the apical cytoplasm of IEC, whereas receptor-bound molecules (e.g. growth factors, sIgA, IgG etc.) are more likely to traverse the cells (Neutra and Kraehenbuhl, 1994). Interestingly, it was reported that cholera toxin, a potent mucosal adjuvant which is taken up by receptor-mediated endocytosis, could activate adenylate cyclase which is basolaterally located by a temperature sensitive vesicular transport mechanism in T84 cells (Lencer *et al.*, 1992). However, no direct apical to basolateral transcytosis was observed. Hence, it is unclear whether this receptor-bound toxin can undergo apical to basolateral transcytosis like other membrane-bound proteins. Thus far, only some invasive bacteria and viruses have been demonstrated to undergo apical to basolateral transcytosis in adult IEC (Levine *et al.*, 1983; Rubin, 1987). Most receptor-bound antigens trancytose from the basolateral to apical side in adult IEC. sIgA is an example of a receptor-bound molecule that undergoes basolateral to apical transcytosis via polymeric Ig receptors (pIgR;

Brandtzaeg, 1981). Several studies have shown that in addition to its role in immune exclusion, IgA transcytosis might help eliminate mucosal immune complexes (Kaetzel *et al.*, 1991) and neutralize viruses intracellularly (Mazanec *et al.*, 1992). In summary, these findings suggest that the fate of internalized antigens depends upon the type of intracellular trafficking they follow in the IEC, which in turn may be determined by the nature of antigen. Several laboratories have reported that IEC can process and present intact soluble antigens like tetanus toxoid (Mayer and Shilen, 1987), ovalbumin (Bland and Warren, 1986a), and keyhole limpet hemocyanin (Kaiserlian *et al.*, 1989) in vitro. Based on previous findings, it is very likely that these soluble proteins are processed in lysosomal compartments and then presented by the IEC to immunocompetent T cells. In this thesis, we have examined the fate of our model antigen, tetanus toxoid after being internalized by the IEC.

PART III: ANTIGEN TRAFFICKING IN INTESTINAL EPITHELIAL CELLS

In conventional APC, the pathway of antigen trafficking dictates the type of T cell response generated. In general, most exogenous antigens follow a class II endocytic pathway, whereas endogenous antigens follow a class I pathway inside the cell. Since our previous studies have demonstrated that IEC can process and present exogenous antigens to $CD8^+$ suppressor T cells, the antigen trafficking pathways in IEC may be distinct.

CLASS I PATHWAY

The classical class I pathway is responsible for the presentation of peptides generated from endogenous antigens including viral, tumor, and self antigens (Braciale, 1992). Endogenous antigens are processed by cytoplasmic proteasomes, which display multicatalytic protease activities. There are two forms of proteasomes, low molecular mass complex (LMP) and 26S proteasome complex (Goldberg and Rock, 1992). Two subunits of LMP (LMP-2 and LMP-7) are encoded by genes located in MHC class II locus (Glynne *et al.*, 1991). Since LMP is polymorphic, a variety of peptides can be generated. The resulting peptides are transported from the cytoplasm to the endoplasmic reticulum (ER) by heterodimeric transporter proteins, TAP-1 and TAP-2, which are also encoded by genes located in the MHC class II locus (Powis *et al.*, 1992). Studies have shown that defects in either TAP-1 or TAP-2 may result in the

formation of unstable class I molecules because the association of peptide with class I heavy chain is required for the correct folding of the heavy chain, which in turn facilitates the binding of β2-microglobulin to the heavy chain (Peters *et al.*, 1991). After the formation of peptide-MHC class I complexes in the ER, these complexes are transferred to the trans-Golgi network where segregation of peptide-MHC class I complexes and class II-invariant chain complexes occurs. The peptide-MHC class I complexes return to the cell surface where they stimulate CD8+ T cells.

CLASS II PATHWAY

Exogenous antigens are known to undergo endosomal uptake, intracellular processing, and coupling to MHC class II molecules before being recycled to the cell surface to be presented to autologous CD4+ T cells. Harding *et al.* (1988) demonstrated that the uptake of radiolabeled β-glucuronidase by macrophages was very rapid. Antigens could be found within the lysosomes of peritoneal macrophages after 15-20 minutes of endocytosis at 37°C. Presentation of processed ^{125}I-*Listeria* to specific T cell hybridomas could occur after 45-60 minutes incubation with ^{125}I-*Listeria* at 37°C (Zeigler and Unanue, 1981). B lymphoma cells showed similar kinetics (Chestnut *et al.*, 1982). Acidic pH and endo/lysosomal proteolytic enzymes (cathepsin D and cathepsin B) are important in antigen processing since lysosomotropic agents, like chloroquine (Zeigler and Unanue, 1982), and protease inhibitors, like leupeptin (Berzofsky *et al.*, 1988), are known to inhibit

processing and subsequent presentation of peptides to T cells. Inside these acidic vesicles, invariant chains are cleaved and antigens are processed. Hence, peptides derived from exogenous antigen could complex with the empty MHC class II molecules. Although there is a consensus about the requirements for intracellular processing and coupling to MHC class II molecules, the actual compartments where antigens are processed to form immunogenic peptides and then complexed to MHC class II molecules are still unresolved. In peritoneal macrophages, utilizing immunoelectron microscopy, Harding *et al.* (1990) showed that 70% of MHC class II were found in prelysosomal "sac-like endocytic structures," 10% in LAMP-1$^+$, cathepsin D$^+$ multivesicular endosomes, and little, if any, in lysosomes. Examining phagocytic processing of *Listeria monocytogenes*, Harding and Geuze (1992) co-localized *Listeria* with MHC class II molecules in tubulo-vesicular lysosomal compartments and phagolysosomes (LAMP-1$^+$, cathepsin D$^+$, M6PR$^-$) by immunoelectron microscopy. Using acid-resistant or acid-sensitive liposome-encapsulated hen egg lysozyme, Harding *et al.* (1991) further demonstrated that antigens (encapsulated in acid-resistant liposomes) processed in lysosomes were presented more efficiently than antigens encapsulated in acid-sensitive liposomes, which released their contents in early endosomal compartments. In addition, Niebling and Pierce (1993) reported that the early endosomal compartment was not the site of antigen processing because antigen that was bound to transferrin and trafficked only through the early endosomes was not processed efficiently in a B lymphoma cell line, CH27. These findings suggest that late

endosomal and lysosomal compartments are the potential sites where peptides are formed and coupled to MHC class II molecules. In human B lymphoblastoid cells, Peters *et al.* (1991) found that most MHC class II molecules were located in special compartments called MIIC compartments (cathepsin D^- and LAMP-1^+; related to lysosomes). They have speculated that MIIC are the potential sites where MHC class II molecules meet peptides rather than the early endosomes (cathepsin D^+) reported in another human B lymphoblastoid cell line by Guagliardi *et al.* (1990). The observation that exogenous antigen entered via transferrin receptors could be processed and presented by B lymphoma cell TA3 supports the concept that the early endosomal compartment is one of the sites of antigen processing (McCloy *et al.*, 1993). Taken together, these data suggest that different APC display different compartmentalization for antigen processing, yet they all activate predominantly $CD4^+$ T cells.

Given the unique environment in the GI tract, the way in which mucosal immune system handles antigen may be distinct from that of the systemic immune system. It is known that antigen which is handled by the M cells is more likely to elicit positive immune responses. Moreover, invasive pathogens that can traverse the intestinal epithelium also induce active immune responses. Although intact soluble proteins have been shown to be capable of traversing the epithelium and entering the circulation, they usually do not elicit active immune responses; rather they induce tolerance. Unfortunately, very little is known about how the mucosal immune

system discriminates in its handling of antigens. Since IEC can serve as unconventional APC which activate a distinct set of T cells ($CD8^+$ suppressor T cells), perhaps the way(s) that IEC handle antigen is distinct. To examine how effective and efficient IEC are as APC and determine whether the ways of antigen handling by the IEC could be responsible for oral tolerance and controlled inflammation in the gut, in this thesis we attempted to compare and contrast the antigen presenting cell properties of conventional APC with those of IEC.

METHODS

Cells

HT29 and DLD-1 are human colonic adenocarcinoma cell lines purchased from the American Type Culture Collection (Rockville, MD). HT29 and DLD-1 cells were maintained at 37°C, 5% CO_2/95% air in RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (Gibco), 1% penicillin & streptomycin (ICN Biomedical, Irvine, CA), and 2mM glutamine (Gibco). The Caco-2 cell line, a gift from Dr. M. W. Musch (University of Chicago), was maintained under similar conditions in DMEM (Gibco) containing 25 mM glucose (Sigma Chemical Co., St. Louis, MO), 20% heat-inactivated fetal calf serum with both essential and non-essential amino acids (Gibco Laboratories), 10 mM HEPES pH 7.4 (Sigma), and 1% penicillin/streptomycin. HT29, DLD-1, and Caco-2 cells were split 1:3 by 1X trypsin/EDTA (Sigma) once a week and cultured in T175 flasks (Nunc, Naperville, IL).

Peripheral blood mononuclear cells were obtained by Ficoll/Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation. Mononuclear cells were collected from the interface and washed three times in PBS. The cells were resuspended in RPMI 1640 and the cell density was adjusted to $5x10^6$/ml. Monocytes were isolated by plastic adherence by plating peripheral mononuclear cells in tissue culture dishes (Falcon, Lincoln, NJ) for 1 h at 37°C.

After removal of unbound cells, adherent monocytes were removed by incubation at 4°C for 1 h followed by vortexing.

Establishment of polarity

Polarity of Caco-2 cells was established as previously described by Hughson *et al.* (1989). $2x10^6$ Caco-2 cells, a cell line known to establish polarity on filters, were seeded onto collagen coated polycarbonate transwell filters (Costar, Cambridge, MA), 24.5 mm in diameter, with 2 ml of medium in the upper chamber and 3 ml in the lower chamber. The medium was replaced every 2 days. The transepithelial resistance of each filter was then measured using the Millicell-ERS resistance system (Millipore, Bedford, MA). Only filters with a resistance greater than 200 ohms-cm^2 were used for experiments. Antigen was added to the upper chamber for varying time periods and antigen uptake was assessed as described below.

Labeling antigen with fluorescein

Antigen (e.g. TT, OVA, KLH, IgG) was dialyzed against conjugation buffer (carbonate buffer, pH 10.0) at 4°C with two changes of buffer. 500 μg of fluorescein isothiocyanate (FITC; 25 mg/ml) was added to 1 ml of dialyzed soluble antigen (7-5 mg/ml) and the mixture was rotated overnight in the dark at 4°C. Unbound FITC was removed by passing the mixture through a sephadex G-25 column (Pharmacia). Fractions were collected and O.D.s at 280 nm and 495 nm were measured by a Spectronic 601 spectrophotometer. Fractions with $O.D._{280}/O.D._{485}$ greater than 0.8 were used and the final protein concentration was adjusted to 2 mg/ml prior to pulsing.

Fluorescence microscopy

To perform the kinetic studies of antigen uptake, IEC grown on glass coverslips or polycarbonate filters were pulsed with 60 μg of FITC-labeled antigen (2 mg/ml) for varying periods at 37°C. This concentration was chosen as optimal for fluorescence analysis in preliminary studies. After incubation, the cells were washed in phosphate buffer saline (PBS) three times and fixed with methanol:acetic acid (3:1) at -20°C for 5 min. The cells were further washed in PBS before mounting with Immu-mount (Shandon, Pittsburgh, PA). Monocytes were treated in the same manner except that they were prepared as cytospin preparations on glass slides before fixation. The slides were then viewed by a Zeiss fluorescence microscope.

Laser scanning confocal microscopy

IEC grown on coverslips or polycarbonate filters were pulsed with 60 μg of FITC-labeled antigen and incubated at 37°C for varying periods. After incubation, the cells were washed in PBS three times, and fixed with methanol:acetic acid (3:1) at -20°C for 5 min. The cells were further washed in PBS before mounting with Immu-mount (Shandon). Monocytes were treated in the same manner except that they were cytospun (100 rpm for 10 min by cytospin, Shandon) onto glass slides before fixation. The slides were then viewed by a Leica fluovert laser scanning confocal microscope at a step position of 1 μm on the X-Y or X-Z axis using the accompanying software. The intensity of fluorescence signals were measured with constant

parameters (e.g. pinhole, voltage, offset) in the same set of experiments. Images were photographed from the computer monitor with Kodak Ektachrome 100Hc or T-Max100 film.

Electron microscopy

The procedure for labeling tetanus toxoid (TT) with colloidal gold particles was based on methods previously described by Parton *et al.* (1987) with slight modifications. 150 mg of TT (Connaught Laboratories, Swiftwater, PA) was dialyzed against distilled water overnight. The pH of 15 nm colloidal gold (Amersham, Arlington Heights, IL) was adjusted to 6.9 before adding to TT. The mixture was stirred for 30 min at room temperature. Successful conjugation was indicated by the absence of a color change from burgundy to gray when a sample of mixture was added to 10% NaCl. The mixture was stabilized by adding 1% carbowax PEG 20,000 (pH 7.4) to a final concentration of 0.05% carbowax. The preparation was washed three times in 0.05% carbowax in PBS, centrifuged at 20,000 rpm in a SW40 rotor, and finally the pellet was resuspended in PBS and stored at 4°C. Preliminary experiments with trace amounts of ^{125}I-TT had shown that TT was adsorbed onto the gold particles since 90% of the radioactivity was recovered in the beads.

To perform kinetic studies of the intracellular trafficking of colloidal gold-labeled TT, HT29 cells grown on chamber slides or Caco-2 cells grown on polycarbonate filters were incubated with

gold-labeled TT for varying periods before fixation with 2% paraformaldehyde, 2.5% glutaraldehyde, 4 mM $CaCl_2$, and 2 mM $MgCl_2$ in 0.1 M sodium cacodylate buffer, pH 7.4 for 45 min. Controls were HT29 or Caco-2 cells pulsed with unconjugated 15 nm gold particles for the same periods and treated in the same manner as the experimental cultures. After 45 min of fixation, the cells were rinsed in 0.1 M sodium cacodylate buffer for 10 min. The cells were then postfixed with 1% osmium tetroxide:3% potassium ferrocyanide (1:1) for 45 min, washed in sodium maleate pH 5.15 twice for 5 min, and stained with 1% uranyl acetate in sodium maleate pH 6.0 for 30 min. After dehydration in an alcohol series (50%, 75%, 90%, 100%; 10 min each), the cells were further dehydrated in 100% ethanol for a second time. Afterwards, the cells were incubated in ethanol:epoxyl resin (EPON; 1:1) for 10 min, 100% EPON for 15 min, and EPON mixture (16 ml of EPON, 11 ml of DDSA, 8 ml of NMA, 0.48 ml of DMP) with three changes in 2 h. In the case of Caco-2, the polycarbonate filters were cut and immersed in propylene oxide for 5 min prior to embedding with the EPON mixture. Finally, the cells were covered with the EPON mixture and incubated at 68°C for 48 h before sectioning with a diamond knife by a Reichert Ultracut ultramicrotome (Valley Cottage, NY). The 600-900 Å sections were mounted on 300 mesh copper grids. The sections were counterstained with 1% uranyl acetate for 5 min and 1% lead citrate for 5 min. Sections were viewed with a Hitachi 7000 transmission electron microscope (Danbury, CT) operated at an accelerating voltage of 75 KV. All chemicals for electron microscopy were

reagent grade and obtained from Electron Microscopy Sciences (Washington, PA).

Immunofluorescence co-localization studies

3-(2,4-dinitroanilino)-3'-amino-N-methyldipropylamine (DAMP) and mouse monoclonal antibody against LAMP-2 (lysosomal marker) as well as rabbit polyclonal antibody against mannose-6-phosphate receptor (M6PR; late endosomal marker) were kindly provided by Dr. W. Dunn (University of Florida). Rabbit polyclonal anti-dinitrophenol (anti-DNP; derivative of DAMP in acidic compartments) antibody was purchased from Molecular Probes (Eugene, OR), while mouse monoclonal antibody against transferrin receptor was purchased from Ortho Diagnostic Systems (Raritan, NJ). Texas red-conjugated swine anti-mouse antibody and Texas red-conjugated donkey anti-rabbit antibody were purchased from Amersham.

Briefly, IEC were incubated with FITC-TT for varying periods of time, after which the cells were rinsed in PBS three times and fixed with methanol:acetic acid (3:1) for 5 min at -20°C. Fixed cells were then incubated with PBS with 5% goat serum and 0.2% Triton X-100 for 15 min prior to incubating with the primary antibody for 1 h, after which the cells were rinsed in PBS three times before incubating with Texas red-conjugated swine anti-mouse or Texas red-conjugated donkey anti-rabbit antibodies. The cells were then rinsed in PBS before mounting with Immu-mount.

To co-localize FITC-TT and DAMP, IEC were incubated with both FITC-TT and 30 μM DAMP. To localize DAMP, the cells were fixed with 4% paraformaldehyde in PBS for 30 min. Afterwards, the cells were washed twice with 50 mM NH_4Cl for 5 min twice prior to permeabilizing with 0.2% Triton X-100 in PBS for 30 min. The cells were then incubated with rabbit polyclonal anti-DNP IgG antibody for 60 min at room temperature. After three washes in PBS, the cells were incubated with Texas red-conjugated donkey anti-rabbit antibodies for 60 min at room temperature. Lastly, the cells were washed, mounted with Immu-mount, and viewed by a fluorescence microscope.

Cytokine pretreatment

IEC grown on glass coverslips were treated with 100 U/ml IFN-γ (Boehringer Mannheim, Indianapolis, IN) for 48 h or 25 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF; R & D Systems, Minneapolis, MN) for 72 h before pulsing with the FITC-labeled antigen. Similarly, monocytes were pretreated with 100 U/ml IFN-γ or 25 ng/ml GM-CSF for 24-48 h. These concentrations were chosen as optimal for enhancing antigen uptake by monocytes. Antigen uptake was quantitated by measuring fluorescence intensity on a per cell basis with the confocal microscope (see above).

Formation of immune complexes and heat-aggregated TT

25 mg/ml of human anti-TT IgG antibody (Miles Inc., Elkhart, IN) was incubated with 8 mg/ml of FITC-TT at 37°C for 1h. The insoluble immune complexes were separated from the soluble immune

complexes by centrifuging at 10,000g for 1h. Before using in the pulsing studies, insoluble immune complexes were resuspended in RPMI and the concentration was adjusted to 2 mg/ml. To prepare anti-TT IgG F(ab)'$_2$ fragments, anti-TT IgG antibody was dialyzed against 0.1M sodium acetate buffer, pH4.5 overnight and then digested with 4% pepsin at 37^{o}C overnight. The F(ab)'$_2$ fragments were then purified by a sephadex G150 column. SDS polyacrylamide gel electrophoresis was used to confirm the fractions containing the anti-TT IgG F(ab)'$_2$ fragments. Anti-TT IgG F(ab)'$_2$/FITC-TT immune complexes were formed in the same manner as the immune complexes with intact anti-TT IgG antibody. To form heat-aggregated FITC-TT, FITC-TT was heated at 68^{o}C for 1h. The heat-aggregated FITC-TT was isolated by centrifuging at 10,000g for 1 h. Before pulsing, heat-aggregated FITC-TT was resuspended in RPMI and the concentration was adjusted to 2 mg/ml.

Subcellular fractionation

4-6x10^7 Caco-2 cells were pulsed with tetanus toxoid (Connaught Lab., Swiftwater, PA) for varying periods at 37^{o}C. Excess tetanus toxoid was washed away with 0.9% NaCl and then with sucrose buffer (250 mM sucrose, 12 mM Tris-HCl pH7.4). The cells were scraped from the flasks in sucrose buffer by a rubber policeman. After disruption the cells at 3800 KPa in a nitrogen cell disruption bomb (Parr Instrument, Moline, Illinois), the cell homogenate was centrifuged for 5 min at 4^{o}C at 270g (SS34 rotor) to separate the supernatant from the pellet (unbroken cells, nuclei, mitochondria etc.). Then the supernatant was centrifuged at 920g for 10 min

(SS34), 2,300g (SS34) for 15 min, and 170,000g for 45 min (A1256) to obtain a crude membrane pellet. As identified by electron microscopy and assayed by various enzyme assays, the crude membrane pellet was composed mainly of endocytic vesicles and membrane-bound organelles (lysosomes, peroxisomes etc.).

Western blot

Fractions isolated from the subcellular fractionation (homogenate, low speed pellet, supernatant, and high speed crude membrane pellet) were separated on a 10% SDS polyacrylamide gel. Samples were transblotted onto a nitrocellulose membrane according to standard methods. The membrane was incubated with the primary rabbit polyclonal antibody against TT (Calbiochem) for 1 h, washed in Tris-buffer saline (0.1% Tween-20, pH 7.6), and then incubated with secondary goat anti-rabbit antibody conjugated to horseradish peroxidase for 1 h. Afterwards, the proteins on the membrane was detected by enhanced chemiluminescence (ECL) detection reagent (Amersham) containing luminol, enhancer, and hydrogen peroxide. Horseradish peroxidase catalyzed the oxidation of luminol in the presence of hydrogen peroxide. Light emitted by luminol (after oxidation) was detected by Kodak X-OMAT imaging film.

Iodination

Ovalbumin (OVA, Sigma) was iodinated by the chloramine T (Fisher Scientific, Pittsburgh, PA) method using 1 mCi ^{125}I (Amersham) and 100 mg OVA. OVA was incubated with chloramine T for 2 min at room temperature before the addition of sodium metabisulphite (3

mg/ml), and KI (20 mg/ml) to stop the reaction. Free ^{125}I was removed by PD-10 column (G25, Pharmacia) or dialysis, and the specific activity of bound ^{125}I was measured by an Iso-Data 20/20 γ-counter (Polymedco). ^{125}I-OVA was eluted in the early fractions and only those fractions which show >80% incorporation were used for experiments.

TCA precipitation

After incubating with ^{125}I-OVA for varying periods at 37°C, HT29 cells or monocytes were lysed with lysis buffer (0.5% NP-40, 150 mM NaCl, 20 mM Tris-HCl, 1mM PMSF, 5 mM iodoacetamide, 20 μg/ml leupeptin, 20 μg/ml aprotinin, pH 7.5). The cell lysate was separated from the broken cells and nuclei by centrifuging at 10,000g for 30 min. Bovine serum albumin (Sigma) was added to the cell lysate to a final concentration of 0.1 mg/ml. An equal volume of ice-cold 20% trichloroacetic acid (TCA; Sigma) was added to the mixture and incubated on ice for 30 min. The suspension was filtered through glass microfiber filter disks (Whatman, Clifton, NJ) under vacuum. The disks were then washed with 5 ml ice-cold 10% TCA. The radioactivity of the disks containing intact ^{125}I-OVA were measured by a Beckman 5500B gamma counter (Beckman, Somerset, NJ). The catabolism of ^{125}I-OVA was measured as follows:

$$\text{\% of intact 125I-OVA radioactivity} = \frac{\text{radioactive counts on the disks}}{\text{radioactive counts in the cell lysate}} \times 100$$

Antigen uptake and trafficking in human intestinal epithelial cells

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Running Title: Antigen traffic in intestinal epithelium

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Abbreviations used in this paper:
APC, antigen presenting cells; Au-TT, gold-labeled tetanus toxoid; CURL, compartment of uncoupling of receptor and ligand; DAMP, 3-(2,4-dinitroanilino)-3'-amino-N-methyldipropylamine; DDSA, dodecenyl succinic anhydride; DMP, 2, 4, 6-tri(dimethylaminomethyl)phenol; DNP, dinitrophenol; EPON, epoxyl resin; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; IEC, intestinal epithelial cells; MHC, major histocompatibility complex; M6PR, mannose-6-phosphate receptor; MVB, multivesicular bodies; NMA, nadic methyl anhydride; PBS, phosphate buffer saline; TT, tetanus toxoid; TF, transferrin.

ABSTRACT

Background: Immune responses in the gut are distinct from those of peripheral lymphoid tissues. Previous studies have documented that intestinal epithelial cells (IEC) are capable of presenting soluble antigens to stimulate MHC restricted antigen primed T cells; however, little is known about the uptake, processing, and presentation of antigens by these nonprofessional antigen presenting cells. The mechanisms of antigen handling by the gut may dictate the type of immune response generated. Methods: Human colonic adenocarcinoma cell lines (HT29, Caco-2, DLD-1) were used as model systems to study antigen uptake and antigen presenting cell properties of IEC. To assess the kinetics of antigen uptake in IEC, fluorescence and confocal microscopy were employed to follow fluoresceinated antigens. To examine the intracellular trafficking of antigen, electron microscopy and immunofluorescence co-localization studies were utilized to follow gold-labeled or fluorescein-labeled tetanus toxoid respectively. Results: Intracellular staining of fluoresceinated tetanus toxoid was not evident in IEC until after 30 min of incubation at 37°C, whereas in monocytes intracellular punctate staining of fluoresceinated tetanus toxoid was evident after 5 min. Furthermore, in contrast to monocytes where the intracellular staining dissipated after 3 h, intracellular staining was still evident in HT29 cells after 5 h. When analyzed by electron microscopy, gold-labeled tetanus toxoid was internalized by HT29 cells and found within endosomes and multivesicular bodies, but not within the lysosomal compartments,

at 60 min. By 2 h, gold-labeled tetanus toxoid was evident in secondary lysosomes. Immunofluorescence co-localization studies using organelle specific markers confirmed that tetanus toxoid followed an endocytic route from early to late endosomes and finally into lysosomal compartments. Similar findings were observed in polarized Caco-2 cells in which antigen was internalized at the apical surface. Conclusions: These results demonstrate that soluble protein antigens such as tetanus toxoid follow an endocytic route in the IEC, and that the kinetics of antigen uptake by intestinal epithelial cells is slower than that of conventional antigen presenting cells. The observation that tetanus toxoid (gold-labeled or fluorescein-labeled) resides in endosomal and lysosomal compartments may have significance with respect to the site(s) of antigen processing in the IEC.

INTRODUCTION

The mechanisms whereby the systemic immune system handles antigens are reasonably well defined. Yet, little is known about how antigens are handled by the mucosal immune system to maintain homeostasis within the gastrointestinal tract, a site where abundant foreign antigens are encountered daily. One manifestation of this tight regulatory network is the induction of oral tolerance, a process of active immune nonresponsiveness, when a foreign antigen is introduced into the host via the oral route (1-3). This process appears to involve the activation of $CD8^+$ suppressor T cells (4, 5) because tolerance to specific antigens can be transferred from orally tolerized animals to naive animals by adoptively transferring splenocytes, especially $CD8^+$ T cells (6, 7). The exact mechanisms of $CD8^+$ suppressor T cell activation in oral tolerance have not been fully elucidated.

Bland *et al.* (8, 9) first demonstrated the ability of intestinal epithelial cells (IEC) to present antigen in a rat model system. They showed that rat enterocytes could process ovalbumin and present ovalbumin peptides to selectively activate antigen-specific $CD8^+$ suppressor T cells (9). Similar functional capacities were described for human IEC using soluble tetanus toxoid (TT) as the antigen (10). T cell responses to tetanus toxoid were blocked by fixing IEC with paraformaldehyde, indicating that processing was essential for antigen presentation. In contrast to the antigen-specific $CD8^+$ suppressor T cells that were activated by rat IEC, $CD8^+$ suppressor T cells activated by human IEC were antigen-nonspecific. Thus far,

the mechanism of CD8+ suppressor T cell activation by IEC has not been defined. One plausible contributing factor may be that antigens are handled differently by IEC compared to conventional antigen presenting cells (APC).

In conventional APC, the pathway of antigen trafficking dictates the type of T cell response generated. Exogenous antigens are known to undergo endosomal uptake, intracellular processing, and coupling to major histocompatibility complex (MHC) class II molecules before being recycled to the cell surface to be presented to autologous CD4+ T cells (11-13). Antigens processed endogenously usually follow a class I pathway, in which antigens are processed by cytoplasmic proteosomes and the resulting peptides are transported to the endoplasmic reticulum (ER) where they form peptide-MHC class I complexes. These complexes return to the cell surface where they stimulate CD8+ T cells (14-16). Since our previous studies have demonstrated that IEC can process and present exogenous antigens to CD8+ suppressor T cells (10), the antigen trafficking pathways in IEC may be distinct. In this study we employed fluorescence and confocal microscopy to assess the uptake of exogenous soluble antigens. To determine whether exogenous antigens follow a class I or class II pathway, we applied electron microscopy and immunofluorescence co-localization assays to examine intracellular trafficking of gold-labeled or fluorescein-labeled antigen in the IEC. We observed that similar to conventional APC, exogenously administered antigens appear to follow a class II pathway in the IEC.

MATERIALS AND METHODS

CELLS

HT29 and DLD-1 are human colonic adenocarcinoma cell lines purchased from American Type Culture Collection (Rockville, MD). HT29 and DLD-1 cells were maintained at 37°C, 5% CO_2/95% air in RPMI 1640 (Sigma, St. Louis, MO) containing 10% heat-inactivated fetal calf serum (Gibco Laboratories, Grand Island, NY), 1% penicillin/streptomycin (ICN Biomedical, Irvine, CA), and 2mM glutamine (Gibco Laboratories). The Caco-2 cell line, a gift from Dr. M. W. Musch (University of Chicago), was maintained under similar conditions in DMEM containing 25 mM glucose (Sigma), 20% heat-inactivated fetal calf serum with both essential and non-essential amino acids (Gibco Laboratories), 10 mM HEPES pH 7.4 (Sigma), and 1% penicillin/streptomycin. HT29, DLD-1, and Caco-2 cells were split 1:3 by 1X trypsin/EDTA (Sigma) once a week and cultured in T175 flasks (Nunc, Naperville, IL).

To isolate monocytes, heparinized venous blood was collected from normal individuals and diluted 1:3 with phosphate buffer saline (PBS), layered on a Ficoll/Hypaque (Pharmacia, Piscataway, NJ) density gradient, and centrifuged at 500g for 30 min. Mononuclear cells were collected from the interface and washed three times in PBS. The cells were then resuspended in RPMI 1640 and the cell density was adjusted to $5x10^6$/ml. Monocytes were isolated by plastic adherence by plating peripheral mononuclear cells in tissue

culture dishes (Falcon, Lincoln, NJ). Adherent monocytes were removed by incubation at 4°C for 1 h followed by vortexing.

ESTABLISHMENT OF POLARITY

Polarity of Caco-2 cells was established as previously described by Hughson *et al.* (17). $2x10^6$ Caco-2 cells, a cell line known to establish polarity on filters, were seeded onto collagen coated polycarbonate transwell filters (Costar, Cambridge, MA), 24.5 mm in diameter, with 2 ml of medium in the upper chamber and 3 ml in the lower chamber. The medium was replaced every 2 days. The transepithelial resistance of each filter was then measured using the Millicell-ERS resistance system (Millipore, Bedford, MA). Only filters with a resistance greater than 200 ohms-cm^2 were used for experiments. Antigen was added to the upper chamber for varying time periods and antigen uptake was assessed as described below.

FLUORESCENCE MICROSCOPY

To label antigens with fluorescein, 2 mg/ml of tetanus toxoid (Connaught Laboratories, Swiftwater, PA) or transferrin (Sigma Chemical Co.) were dialyzed against conjugation buffer (carbonate buffer, pH 10.0) at 4°C with two changes of buffer. 20 µl of fluorescein isothiocyanate (FITC; Sigma Chemical Co.) in DMSO (25 mg/ml; Sigma Chemical Co.) was added to 1 ml of dialyzed soluble antigen (5 - 7 mg/ml) and the mixture was rotated overnight in the dark at 4°C. Unbound FITC was removed by passing the mixture through a Sephadex G-25 column (Pharmacia). Fractions were collected and O.D. 280 nm and 495 nm were measured by a Spectronic

601 spectrophotometer (Milton Roy Company, Rochester, NY). Fractions with $O.D._{280}/O.D._{495}$ greater than 0.8 were used for pulsing.

To perform the kinetic studies of antigen uptake, IEC grown on glass coverslips or polycarbonate filters were pulsed with 60 μg of FITC-TT (2 mg/ml) for varying periods at 37°C. This concentration was chosen as optimal for fluorescence analysis in preliminary studies. After incubation, the cells were washed in PBS three times and fixed with methanol:acetic acid (3:1) at -20°C for 5 min. The cells were further washed in PBS before mounting with Immu-mount (Shandon, Pittsburgh, PA). Monocytes were treated in the same manner except that they were prepared as cytospin preparations on glass slides before fixation. The slides were then viewed by a Zeiss fluorescence microscope.

LASER SCANNING CONFOCAL MICROSCOPY

IEC were treated in the same manner as for the fluorescence microscopic studies. The slides were viewed and sectioned with a Leica fluovert laser scanning confocal microscope (Deerfield, IL) at a step position of 1 μm on the X-Y or X-Z axis. Images were photographed from the computer monitor with Kodak Ektachrome 100Hc or T-Max 100 film.

ELECTRON MICROSCOPY

The procedure for labeling tetanus toxoid (TT) with colloidal gold particles was based on methods previously described by Parton *et al.* (18) with slight modifications. 150 mg of tetanus toxoid (Connaught Laboratories, Swiftwater, PA) was dialyzed against distilled water overnight. The pH of 15 nm colloidal gold (Amersham, Arlington Heights, IL) was adjusted to 6.9 before adding to TT. The mixture was stirred for 30 min at room temperature. Successful conjugation was indicated by the absence of a color change from burgundy to gray when a sample of mixture was added to 10% NaCl. The mixture was stabilized by adding 1% carbowax PEG 20,000 (pH7.4) to a final concentration of 0.05% carbowax. The preparation was washed three times in 0.05% carbowax in PBS, centrifuged at 20,000 rpm in a SW40 rotor, and finally the pellet was resuspended in PBS and stored at 4°C. Preliminary experiments with trace amounts of ^{125}I-TT had shown that TT was adsorbed onto the gold particles since 90% of the radioactivity was recovered in the beads.

To perform kinetic studies of the intracellular trafficking of colloidal gold-labeled TT, HT29 cells grown on chamber slides or Caco-2 cells grown on polycarbonate filters were incubated with gold-labeled TT for varying periods before fixation with 2% paraformaldehyde, 2.5% glutaraldehyde, 4 mM $CaCl_2$, and 2 mM $MgCl_2$ in 0.1 M sodium cacodylate buffer, pH 7.4 for 45 min. Controls were HT29 or Caco-2 cells pulsed with unconjugated 15 nm gold particles

for the same periods and treated in the same manner as the experimental cultures. After 45 min of fixation, the cells were rinsed in 0.1 M sodium cacodylate buffer for 10 min. The cells were then postfixed with 1% osmium tetroxide:3% potassium ferrocyanide (1:1) for 45 min, washed in Na maleate pH 5.15 twice for 5 min, and stained with 1% uranyl acetate in Na maleate pH 6.0 for 30 min. After dehydration in an alcohol series (50%, 75%, 90%, 100%; 10 min each), the cells were further dehydrated in 100% ethanol for a second time. Afterwards, the cells were incubated in ethanol:epoxyl resin (EPON; 1:1) for 10 min, 100% EPON for 15 min, and EPON mixture (16 ml of EPON, 11 ml of DDSA, 8 ml of NMA, 0.48 ml of DMP) with three changes in 2 h. In the case of Caco-2, the polycarbonate filters were cut and immersed in propylene oxide for 5 min prior to embedding with EPON mixture. Finally, the cells were covered with EPON mixture and incubated at 68°C for 48 h before sectioning with a diamond knife by a Reichert Ultracut ultramicrotome (Valley Cottage, NY). The 600-900 Å sections were mounted on 300 mesh copper grids. The sections were counterstained with 1% uranyl acetate for 5 min and 1% lead citrate for 5 min. Sections were viewed with a Hitachi 7000 transmission electron microscope (Danbury, CT) operated at an accelerating voltage of 75 KV. All chemicals for electron microscopy were reagent grade and obtained from Electron Microscopy Sciences (Washington, PA)

IMMUNOFLUORESCENCE CO-LOCALIZATION STUDIES

3-(2,4-dinitroanilino)-3'-amino-N-methyldipropylamine (DAMP), and mouse monoclonal antibody against LAMP-2 (lysosomal marker) as well as rabbit polyclonal antibody against mannose-6-phosphate receptor (M6PR; late endosomal marker) were kindly provided by Dr. W. Dunn (University of Florida). Rabbit polyclonal anti-dinitrophenol (anti-DNP; derivative of DAMP in acidic compartments) antibody was purchased from Molecular Probes (Eugene, OR); mouse monoclonal antibody against the transferrin receptor was purchased from Ortho Diagnostic Systems (Raritan, NJ); Texas red-conjugated swine anti-mouse antibody and Texas red-conjugated donkey anti-rabbit antibody were purchased from Amersham.

Briefly, IEC were incubated with FITC-TT for varying periods of time, after which the cells were rinsed in PBS three times and fixed with methanol:acetic acid (3:1) for 5 min at -20°C. Fixed cells were then blocked with PBS containing 5% goat serum and 0.2% Triton X-100 for 15 min prior to incubating with the primary antibody (e.g. anti-LAMP-2, anti-M6PR, anti-transferrin receptor) for 1 h. The cells were then rinsed in PBS three times before incubating with Texas red-conjugated swine anti-mouse or Texas red-conjugated donkey anti-rabbit antibodies. The cells were then rinsed in PBS before mounting with Immu-mount.

To co-localize FITC-TT and DAMP, IEC were incubated with both FITC-TT and 30 mM DAMP. To localize DAMP, the cells were fixed with 4% paraformaldehyde in PBS for 30 min. Afterwards, the cells

were washed twice with 50 mM NH_4Cl for 5 min twice prior to permeabilization with 0.2% Triton X-100 in PBS for 30 min. The cells were then incubated with rabbit polyclonal anti-DNP IgG antibody for 60 min at room temperature. After three washes in PBS, the cells were incubated with Texas red-conjugated donkey anti-rabbit antibodies for 60 min at room temperature. Lastly, the cells were washed, mounted with Immu-mount, and viewed by a fluorescence microscope.

RESULTS

ANTIGEN UPTAKE BY IEC LINES

Since human colonic adenocarcinoma cell lines such as HT29, Caco-2, and DLD-1 have been documented to possess functional properties comparable to their non-malignant counterparts, we used them as model systems to study antigen handling. To determine the effectiveness of nonspecific soluble antigen uptake, intestinal epithelial cell lines, grown on coverslips, were pulsed with fluoresceinated tetanus toxoid (FITC-TT) for 60 min at 37°C and then examined by fluorescence microscopy. The results of this staining are shown in Fig. 1. An intracellular speckled staining pattern was observed in all three cell lines. The diverse staining patterns might reflect the distinct distribution of endosomal compartments in different cell lines. Formaldehyde-fixed cells (Fig. 1D) and cells incubated at 4°C (Fig. 1E) showed no evidence of antigen uptake, suggesting that internalization is an active process. We also found that chloroquine pretreatment (100 mM x 1 h) had no effect on the uptake of TT in IEC lines (data not shown), suggesting that the uptake of TT by IEC is via non-receptor-mediated endocytosis.

KINETICS OF ANTIGEN UPTAKE DETERMINED BY FLUORESCENCE MICROSCOPY

To study the efficiency of antigen uptake by IEC compared to monocytes, we examined the kinetics of antigen uptake. HT29 cells

grown on glass coverslips and isolated monocytes cytospun onto glass slides were pulsed with FITC-TT and incubated at 37°C for varying periods, after which they were washed with PBS, fixed with methanol:acetic acid (3:1), and examined by fluorescence microscopy. As shown in Fig. 2, the uptake of FITC-TT was much faster in monocytes than that seen in HT29 cells (Fig. 3). Extensive speckled fluorescence signals were evident at 5 min (Fig. 2B) and started to decrease by 60 min (Fig. 2D), perhaps due to processing or recycling of FITC-TT to the membrane. These results concur with the rate of antigen uptake and processing by macrophages reported in the literature (19-21). In HT29, punctate fluorescence signals were not evident until 30 min at 37°C (Fig. 3C). Hence, the uptake of TT by IEC appears to be slower than that of monocytes. No diminution of fluorescence signals was observed in HT29 even after 2 h of incubation (Fig. 3E). This observation was distinct from that seen in monocytes where the fluorescence signals dissipated after 3 h (Fig. 2E), suggesting that either the recycling of endocytic vesicles or the processing of TT by IEC is slower compared with monocytes. A series of pulse-chase experiments were performed in which HT29 cells were pulsed with FITC-TT for 60 min at 37°C, washed with PBS to remove unbound FITC-TT, and chased at 37°C for varying periods. In contrast to monocytes, fluorescence signals were still noted in HT29 after a 5 h chase (Fig. 3F). These findings suggest that the rate of antigen handling (recycling of endocytic vesicles or processing) by IEC is distinct from that of monocytes. Similar kinetics was observed in other IEC lines, DLD-1 and Caco-2 (data not shown). HT29 cells which were kept in suspension also displayed

similar kinetics of antigen uptake (data not shown). However, the rate of antigen uptake appeared to depend upon the manner of antigen uptake. Receptor-mediated endocytosis was noted to be much more efficient and rapid than non-receptor-mediated endocytosis. In the case of transferrin (TF), which is internalized via TF receptors, intracellular FITC-TF fluorescence signals were evident in the IEC after 15 min incubation at 37°C (data not shown).

KINETICS OF ANTIGEN UPTAKE DETERMINED BY CONFOCAL MICROSCOPY

We employed confocal microscopy to obtain a better assessment of the kinetics and dynamic localization of antigen uptake. HT29 cells grown on coverslips were pulsed with FITC-TT and incubated at 37°C for varying periods before fixation with methanol:acetic acid (3:1). The cells were then sectioned by confocal microscopy to provide lateral views of fluorescence staining (Fig. 4). Punctate fluorescence signals were observed inside the cells at 30 min (Fig. 4A), confirming the data obtained by conventional fluorescence microscopy. Staining patterns were highly suggestive of endosomal uptake of FITC-TT. There appeared to be polar trafficking of FITC-TT within the HT29 cells (Figs. 4B, C), although these cells do not grow as polar monolayers.

POLAR TRANSPORT OF ANTIGEN DETERMINED BY CONFOCAL MICROSCOPY

To examine polar transport of FITC-TT more directly, polarized Caco-2 cells grown on polycarbonate filters were used. When FITC-TT was applied apically, punctate fluorescence signals were observed inside the cells after 30 min incubation at 37°C similar to HT29 (data not shown). Interestingly, after incubating Caco-2 cells with FITC-TT for 2 h, fluorescence signals remained in the apical cytoplasm (Fig. 5), whereas FITC-TT that was pulsed basolaterally for 2 h was transcytosed to the apical side (Fig. 6). These findings suggest a polarized transport process that may be site dependent. A similar observation was also reported by Hughson *et al.* who examined the endocytic pathways in polarized Caco-2 cells (22).

INTRACELLULAR TRAFFICKING OF ANTIGEN

To more carefully determine the fate of antigens following their internalization, we conducted kinetic studies to follow the intracellular trafficking of colloidal gold-labeled TT (Au-TT) utilizing electron microscopy. HT29 cells grown on chamber slides were pulsed with Au-TT or unconjugated colloidal gold particles, incubated at 37°C for varying periods, fixed, postembedded, and examined by electron microscopy (Fig. 7). At 5 min, most of the Au-TT particles were bound to the cytoplasmic processes and remained on the cell surface (Fig. 7A). Very few Au-TT particles were observed inside the cells. By 30 min, Au-TT was evident in early endosomes and endosomal compartments that resemble the compartment of uncoupling of receptor and ligand (CURL) (Fig. 7B). After 45 min incubation, multivesicular bodies (MVB) were labeled

with Au-TT (Figs. 7C, D), and Au-TT was found in secondary lysosomes after 2 h incubation (Fig. 7E). Minimal uptake of unconjugated gold particles was noted in controls. Similar findings were observed in polarized Caco-2 cells grown on polycarbonate filters (Fig. 8). Au-TT was endocytosed at the base of the microvilli (Fig. 8B) and found in MVB as well as lysosomes (primary and secondary) in the apical cytoplasm above the nucleus (Fig. 8C) with kinetics similar to HT29 cells. These vesicles did not traffic to the basolateral surface. These results demonstrate that soluble protein antigen follows an endocytic route from early endosomes to late endosomes, and subsequently into the lysosomal compartments inside the IEC. Class II MHC molecules have been reported to be present in MVB and lysosomes in the IEC (23, 24), which, interestingly, were also the compartments found to be labeled with Au-TT in our studies.

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One potential pitfall of electron microscopy is that there might be dissociation of TT from the gold particle within the endocytic vesicles and thus we might follow the gold label rather than the protein itself. Furthermore, since electron microscopy could only identify the vesicular compartments morphologically, we performed immunofluorescence co-localization studies of FITC-TT with markers of endosomal and lysosomal compartments to more clearly define the intracellular antigen trafficking. HT29 cells grown on coverslips were allowed to endocytose FITC-TT for various periods,

after which the cells were fixed with methanol:acetic acid (3:1) and incubated with primary antibodies against endosomal (TF receptor, M6PR) or lysosomal (LAMP-2) proteins. To localize acidic compartments, HT29 cells were co-incubated with FITC-TT and DAMP (30 μM), which has been reported to accumulate in acidic vesicles. At 30 min, most FITC-TT was evident in early endosomal compartments labeled with TF receptor (Fig. 9). At 60 min, FITC-TT appeared in late endosomal compartments which were DAMP and M6PR positive (Figs. 10 and 11) but TF receptor negative (data not shown). No FITC-TT was observed in LAMP-2 positive lysosomal compartments at this time point (Fig. 12). When FITC-TT was pulsed for 1 h and chased for 3 h, most of the FITC-TT was localized in lysosomal compartments which were DAMP (Fig. 13) and LAMP-2 (Fig. 14) positive; FITC-TT was no longer observed in transferrin receptor positive early endosomes (Fig. 15). Similar observations were made in Caco-2 cells (data not shown). These findings support the data obtained from the electron microscopic studies, suggesting that FITC-TT follows a classical class II endocytic pathway in the IEC.

DISCUSSION

In order to initiate an immune response, exogenous antigens are internalized, processed into small peptides and presented in the context of MHC class II molecules to T cells (11-13). In macrophages and B cells, the classical class II pathway of intracellular trafficking has been extensively studied (25-27). Although the exact site(s) where antigen processing occurs is unresolved and the endocytic compartments are poorly defined, it is generally accepted that endosomal and lysosomal compartments are the sites of antigen processing and complexing of peptides to MHC class II molecules. Since normal class II antigen positive IEC are known to stimulate $CD8^+$ T cells, we examined the intracellular trafficking of a soluble exogenous antigen in the IEC to determine if it followed a class I or class II pathway. Consistent with previous findings in the conventional APC, the electron microscopic and immunofluorescence co-localization studies showed that our model antigen (TT) followed a class II endocytic pathway inside the IEC. Gold-labeled or fluorescein-labeled TT was endocytosed and detected in early endosomes, then in late endosomes (e.g. MVB), and subsequently in secondary lysosomes. Studies have shown that IEC express both MHC class II molecules (28-30) and invariant chain (31). Functionally, these MHC class II molecules can present antigens to T cells (e.g. Keyhole limpet hemocyanin to a $CD4^+$ T cell hybridoma) (32). Interestingly, MHC class II molecules have been reported to be expressed on the basolateral membranes, in multivesicular bodies, and lysosomes situated in the apical

cytoplasm of IEC (23, 24). These are also the compartments where internalized gold-labeled or fluorescein-labeled TT was observed in HT29 and Caco-2 cells. Such findings suggest that these compartments could be the potential sites of antigen processing and complexing of peptides with MHC class II molecules in the IEC. The MHC class II molecules at the basolateral membranes might then present the peptides to adjacent T cells in the intraepithelial space or in the lamina propria. Since acidic endosomal and lysosomal compartments are the proposed sites of antigen processing in conventional APC, locating TT in these compartments may be significant with respect to confining antigen processing and presentation to the IEC. In vivo, if most soluble protein antigens are taken up, processed, and presented by the IEC, the conventional APC (macrophages and dendritic cells) in the lamina propria might have less of a chance to sample sufficient antigens to elicit active immune responses. This might explain why most soluble protein antigens administered orally are known to induce a tolerant state. If uptake, processing and presentation of antigen by the IEC are important to this phenomenon, the mechanism of antigen trafficking may be critical. We have demonstrated that TT, a soluble protein antigen taken up by fluid phase endocytosis, trafficked to the late endosomal and lysosomal compartments in contrast to receptor-bound antigens (e.g. growth factors, IgG) or invasive organisms (e.g. bacteria, virus) which can undergo apical to basolateral transcytosis in the IEC. In adult IEC, receptor-bound antigens such as IgA/polymeric Ig receptor complexes usually transcytose from the basolateral to apical surfaces. Recent data from Mazanec's group

show that IgA anti-Sendai virus antibody can be taken up basolaterally and co-localized in compartments where active viral replication occurs, thus resulting in viral neutralization (33). Different trafficking pathways might therefore dictate distinct immunologic responses.

In polarized Caco-2 cells, fluorescein-labeled TT applied apically did not traffic to the basolateral surface. Based on the data obtained from electron microscopic studies, gold-labeled TT was found to reside in lysosomes located in the apical cytoplasm and did not transcytose to the basolateral region. Similar observations were made by Cornell *et al.* when they examined the transport of intact proteins across the rat intestinal epithelium in vivo (34). Horseradish peroxidase (HRP) was found in endosomes and secondary lysosomes which were located in the apical cytoplasm. Since HRP was also evident in the intercellular spaces between adjacent absorptive cells, they suggested that at the level of nucleus, HRP was transported into the intercellular spaces, basal lamina, and finally to the lamina propria. In addition, Lencer *et al.* reported that by an unknown temperature sensitive vesicular transport mechanism, cholera toxin applied apically activated adenylate cyclase in the basolateral membranes. However, they did not directly show that fluorescein-labeled cholera toxin, applied apically to polarized T84 cells, trafficked to the basolateral regions (35). Thus, whether cholera toxin is transcytosed through the IEC is unclear. If cholera toxin can undergo apical to basolateral transcytosis, perhaps it can reach the lamina propria and elicit

active immune responses. Although this might help explain its potent immunogenicity, no evidence exists to support this postulate. Similar to our findings on the polar transport of TT in polarized Caco-2 cells, Hughson *et al.* did not observe any apical to basolateral transcytosis of gold-labeled concanavalin A which was applied apically to polarized Caco-2 (22). However, transferrin peroxidase conjugate that was applied basolaterally was co-localized with gold-labeled concanavalin A in endosomes located in the apical cytoplasm, suggesting that apical and basolateral endosomes can meet in common endosomal compartments in the apical cytoplasm. Thus far, most direct transcytotic pathways for soluble proteins (e.g. IgG and growth factors) from the apical surface to basolateral regions have only been observed in neonatal rat IEC and rarely in adult IEC (36, 37). Only invasive bacteria and viruses have been shown to traverse the IEC from the apical to the basolateral sides (38, 39). Since they gain access to the lamina propria, they can be taken up and processed by the conventional APC located there to elicit active mucosal immune responses.

Although IEC are capable of internalizing soluble antigens (TT, TF), the kinetics of antigen uptake via non-receptor-mediated endocytosis by IEC was slower than that of conventional APC. Intracellular fluorescence staining was only evident in IEC after 30 min incubation at 37°C. In monocytes, antigen uptake was a much more rapid process. By 3 h, the intracellular fluorescence staining had dissipated in monocytes, whereas intracellular fluorescence staining persisted in IEC even after a 5 h period. Furthermore, by

electron microscopy, gold-labeled TT was only evident in secondary lysosomes by 2 h, in contrast to macrophages where antigens have been reported in lysosomes by 10-20 min (19, 20). These findings suggest that the endocytic trafficking in IEC is slower than that in monocytes. However, since the intestinal epithelial cell lines used in the studies reported here were derived from colonic adenocarcinoma cells, the actual kinetics of antigen uptake and processing might be different in vivo. We have preliminarily examined the kinetics of antigen uptake and processing in freshly isolated IEC and they appear to be similar although lesser quantitatively. Bland *et al.* have reported that rat IEC are less efficient in antigen processing than conventional APC (40). When comparing lysates from enterocytes with lysates from splenocytes after pulsing with ovalbumin, minimal processing of antigen was observed in rat IEC. To further explore these events in humans, subcellular fractionation is currently being employed to isolate processed TT in the endosomal and lysosomal fractions.

One interesting feature concerning the antigen presenting properties of IEC relates to the observation that despite the expression of class II antigens, normal IEC selectively activate $CD8^+$ suppressor T cells when co-cultured with autologous T cells in the presence of antigen in vitro (9, 10). Conventionally, $CD8^+$ T cells are activated by antigens processed via the endogenous route in a classical class I pathway (15, 16). In this pathway, peptides bound to MHC class I molecules are derived from endogenous antigens processed by proteosomes in the cytoplasm. These peptides are then transferred

into the ER where the antigenic peptides complex to and stabilize the assembly of MHC class I molecules. The MHC class I-peptide complexes are then transported to the cell surface via the Golgi and trans-Golgi network (41, 42). However, the present studies show that exogenous antigen (TT) follows an endocytic pathway rather than a conventional class I pathway in the IEC. Since IEC have been demonstrated to activate $CD8^+$ T cells, one possible explanation is that exogenous antigens might be processed by an alternative class I pathway in the IEC. Evidence to support this possibility comes from Pfeifer *et al.* who demonstrated that bacteria expressing ovalbumin were phagocytosed by peritoneal macrophages into endosomal compartments (43). In spite of the fact that the bacteria was processed in the endosomal compartments and not in the cytoplasm, peptides derived from the bacteria were able to be presented by MHC class I molecules to elicit a $CD8^+$ T cell response. This pathway was demonstrated to be more efficient for particulate antigens and high concentrations of soluble antigen, a scenario consistent with what might be present in the gut. In this unconventional pathway, bacterial antigens processed in the endosomal and lysosomal compartments were recycled back to the cell surface where they bound to empty MHC class I molecules. However, the $CD8^+$ T cells used in these studies were antigen-specific hybridomas and thus, may not reflect a comparable system in which one might account for the $CD8^+$ suppressor T cell activation induced by the IEC.

In the present study, human intestinal epithelial cell lines were utilized as model systems to examine antigen uptake and trafficking. Although intestinal epithelial cell lines might differ from their non-malignant counterparts, they allow for the establishment of optimal conditions for future studies on freshly isolated normal IEL. In conclusion, we have demonstrated that intestinal epithelial cell lines are capable of internalizing exogenous antigens, which follow a classical class II endocytic pathway. Given that IEC selectively activate $CD8^+$ suppressor T cells, clearly, there is a dichotomy in antigen handling between the IEC and conventional APC. Dissection of these differences may contribute to a better understanding of the role of IEC in gut mucosal immune responses.

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FIGURE LEGENDS

Figure 1. Uptake of FITC-TT by HT29 (A), Caco-2 (B), and DLD-1 (C). Intestinal epithelial cells grown on coverslips were incubated with 60 μg of FITC-TT (2 mg/ml) for 60 min at 37°C and washed in PBS prior to fixation with methanol:acetic acid (3:1). Different fluorescence staining patterns were observed. Controls were HT29 cells fixed with 3.7% formaldehyde (D) or incubated at 4°C during endocytosis (E). No antigen uptake was evident in either control. (X100.)

Figure 2. Kinetic studies of FITC-TT uptake by monocytes. Monocytes were pulsed with 60 μg of FITC-TT for 0 min (A), 5 min (B), 30 min (C), 60 min (D), and 3 h (E) at 37°C prior to fixation with methanol:acetic acid (3:1). Intracellular fluorescence staining was evident at 5 min (B) and diminished by 3 h (E). (X100.)

Figure 3. Kinetic studies of FITC-TT uptake by HT29. HT29 cells grown on coverslips were pulsed with 60 μg of FITC-TT for 15 min (A), 30 min (B), 60 min (C), and 2 h (D) at 37°C prior to fixation with methanol:acetic acid (3:1). HT29 cells were also pulsed with 60 μg of FITC-TT for 60 min, washed, and chased for 5 h (E) prior to fixation. Intracellular fluorescence staining was evident at 30 min and no significant diminution of signal was noted even after 5 h. (X100.)

Figure 4. Lateral views of HT29 pulsed with FITC-TT for 30 min (A), 60 min (B), and 2 h (C) at 37°C generated by laser scanning confocal microscopy confirmed endosomal uptake of FITC-TT. The cells were subjected to the same methods used in fluorescence microscopy. (X100.)

Figure 5. Confocal micrographs of Caco-2 pulsed with FITC-TT apically for 2 h. A shows the XZ section of the cells. Fluorescence signals stayed in the apical cytoplasm. B, C and D show the representative XY sections of the cells from the apical surface (B), the middle of the cell (C), and the basal surface (D) respectively. Fluorescence signals were mostly evident in the apical cytoplasm (B, C) and significant less at the basal level (D). (X100.)

Figure 6. Confocal micrographs of Caco-2 pulsed with FITC-TT basally for 2 h. A shows the XZ section of the cells. Fluorescence signals were evident at both the basolateral and apical cytoplasm. B, C, and D show the representative XY sections of the cells from the apical surface (B, C) to the basal surface (D) respectively. Fluorescence signals were evident in all sections (B, C, and D). (X100.)

Figure 7. Electron micrographs of HT29 pulsed with Au-TT for 5 min (A, X20K), 30 min (B, X20K), 45 min (C, X50K), 60 min (D, X50K), and 2 h (E, X25k) at 37°C, fixed, and postembedded. Au-TT (arrows) was evident on the cytoplasmic processes (A), in early endosomes (B),

CURL (B), MVB (C and D), and secondary lysosomes (E) at sequential time points.

Figure 8. Electron micrographs of polarized Caco-2 cells grown on polycarbonate filters (A, X4K) and pulsed apically with Au-TT for 45 min (B, X20K) and 3 h (C, X20K). Au-TT (arrows) was evident at the base of microvilli, and in MVB by 45 min (B), and in MVB, primary lysosomes and secondary lysosomes by 3 h (C).

Figure 9. Intracellular co-localization of FITC-TT and TF receptor in HT29. HT29 cells grown on coverslips were incubated with 60 μg FITC-TT for 30 min at 37°C prior to fixation with methanol:acetic acid (3:1). The cells were then washed and processed for indirect immunofluorescence co-localization studies with anti-TF receptor antibody (Texas red). Cellular compartments containing TF receptor appear red (A), while those containing FITC-TT appear green (B). At 30 min, most FITC-TT co-localized with TF receptor and appear yellow (arrows; C), suggesting that FITC-TT resided in early endosomal compartments at 30 min. (X100).

Figure 10. Intracellular co-localization of FITC-TT and DAMP in HT29. HT29 cells grown on coverslips were incubated with FITC-TT and DAMP for 60 min at 37°C prior to fixation with methanol:acetic acid (3:1). The cells were then washed and processed for indirect immunofluorescence co-localization studies with anti-DNP antibody (Texas red). Cellular compartments containing DAMP appear red (A), while those containing FITC-TT appear green (B). At 60 min, most

FITC-TT co-localized with DAMP and appear yellow (C; arrows), suggesting that FITC-TT localized in acidic compartments at 60 min. (X100.)

Figure 11. Intracellular co-localization of FITC-TT and M6PR in HT29. HT29 cells grown on coverslips were incubated with 60 μg FITC-TT for 60 min at 37°C prior to fixation with methanol:acetic acid (3:1). The cells were then washed and processed for indirect immunofluorescence co-localization studies with anti-M6PR antibody (Texas red). Cellular compartments containing M6PR appear red (A), while those containing FITC-TT appear green (B). At 60 min, most FITC-TT co-localized with M6PR and appear yellow (arrows; C), suggesting that FITC-TT localized in late endosomal compartments at 60 min. (X100.)

Figure 12. Intracellular co-localization of FITC-TT and LAMP-2 in HT29. HT29 cells grown on coverslips were incubated with 60 μg FITC-TT for 60 min at 37°C prior to fixation with methanol:acetic acid (3:1). The cells were then washed and processed for indirect immunofluorescence co-localization studies with anti-LAMP-2 antibody (Texas red). Cellular compartments containing M6PR appear red (A), while those containing FITC-TT appear green (B). At 60 min, most FITC-TT did not co-localize with LAMP-2 (C), suggesting that FITC-TT did not localize in lysosomal compartments at 60 min. (X100.)

Figure 13. Intracellular co-localization of FITC-TT and DAMP in HT29 in pulse-chase studies. HT29 cells grown on coverslips were pulsed with 60 μg FITC-TT and DAMP for 60 min at 37°C and chased for 3 h in RPMI containing 30 μM DAMP. The cells were then fixed with methanol:acetic acid (3:1), washed, and processed for indirect immunofluorescence co-localization studies. Cellular compartments containing DAMP appear red (A), while those containing FITC-TT appear green (B). After a 3 h chase, most FITC-TT co-localized with DAMP and appear yellow (arrows; C), suggesting that FITC-TT remained in acidic compartments. (X100.)

Figure 14. Intracellular co-localization of FITC-TT and LAMP-2 in HT29 in pulse-chase studies. HT29 cells grown on coverslips were pulsed with 60 μg FITC-TT for 60 min at 37°C and chased for 3 h in RPMI. The cells were then fixed with methanol:acetic acid (3:1), washed, and processed for indirect immunofluorescence co-localization studies with anti-LAMP-2 antibody (Texas red). Cellular compartments containing LAMP-2 appear red (A), while those containing FITC-TT appear green (B). After a 3 h chase, most FITC-TT co-localized with LAMP-2 and appear yellow (arrows; C), suggesting that most FITC-TT localized in LAMP-2+ lysosomal compartments at 3 h. (X100.)

Figure 15. Intracellular co-localization of FITC-TT and TF receptor in HT29 in pulse-chase studies. HT29 cells grown on coverslips were pulsed with 60 μg FITC-TT for 60 min at 37°C and chased for 3 h in RPMI. The cells were then fixed with methanol:acetic acid (3:1),

washed, and processed for indirect immunofluorescence co-localization studies with anti-TF receptor antibody (Texas red). Cellular compartments containing TF receptor appear red (A), while those containing FITC-TT appear green (B). At 60 min, most FITC-TT did not co-localize with TF receptor (C), suggesting that FITC-TT did not localize in early endosomal compartments at 3 h. (X100.)

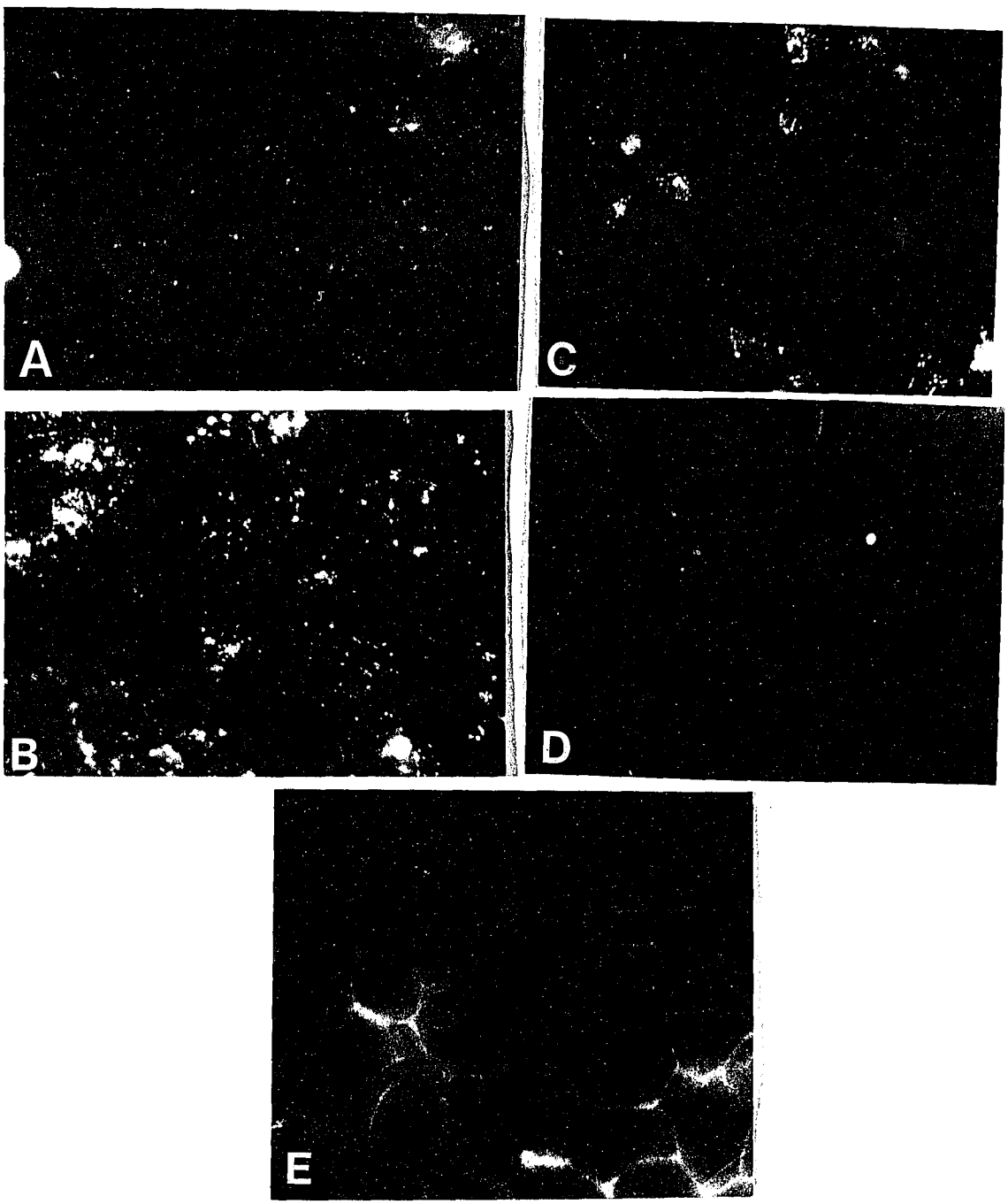
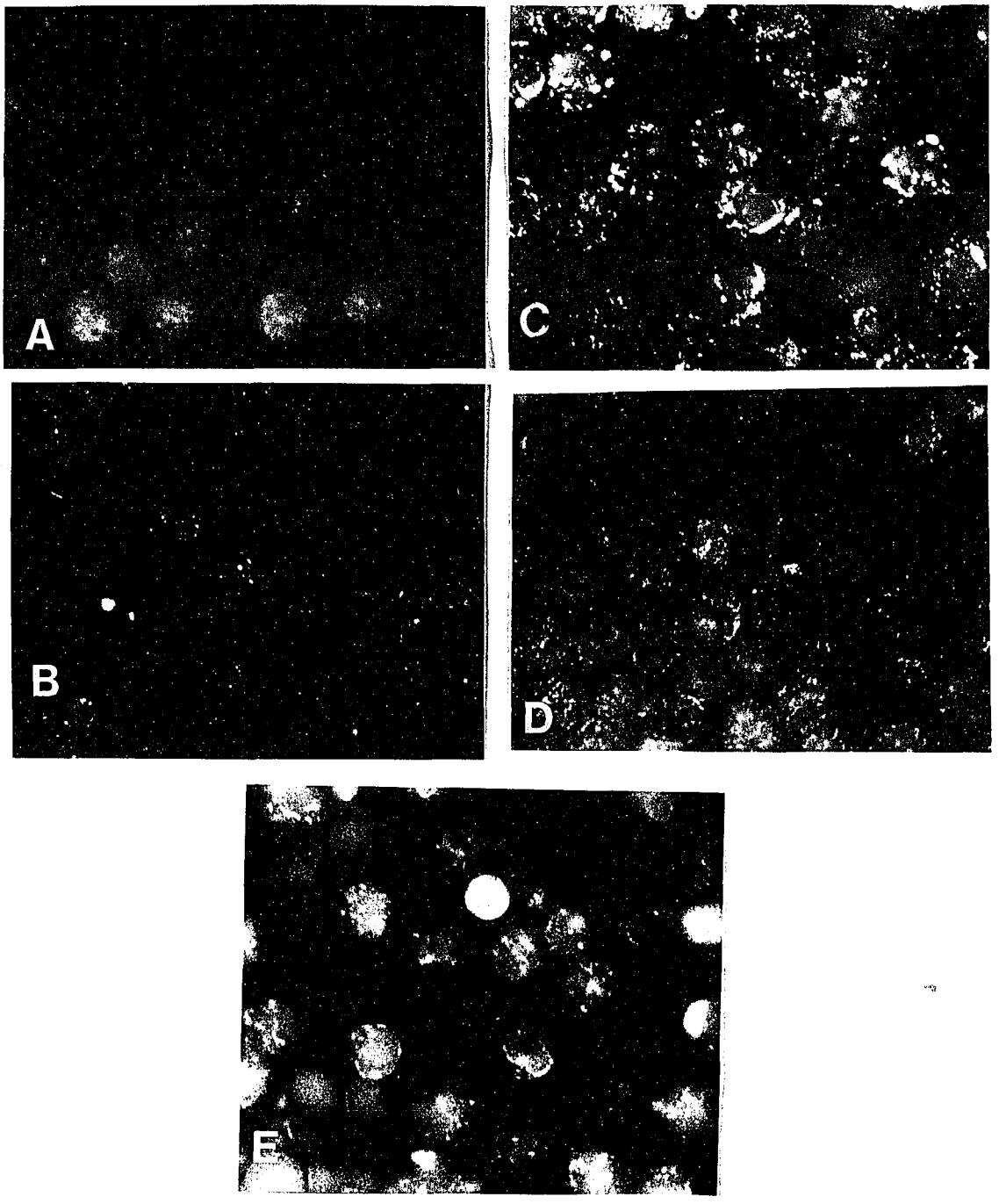


Figure 1



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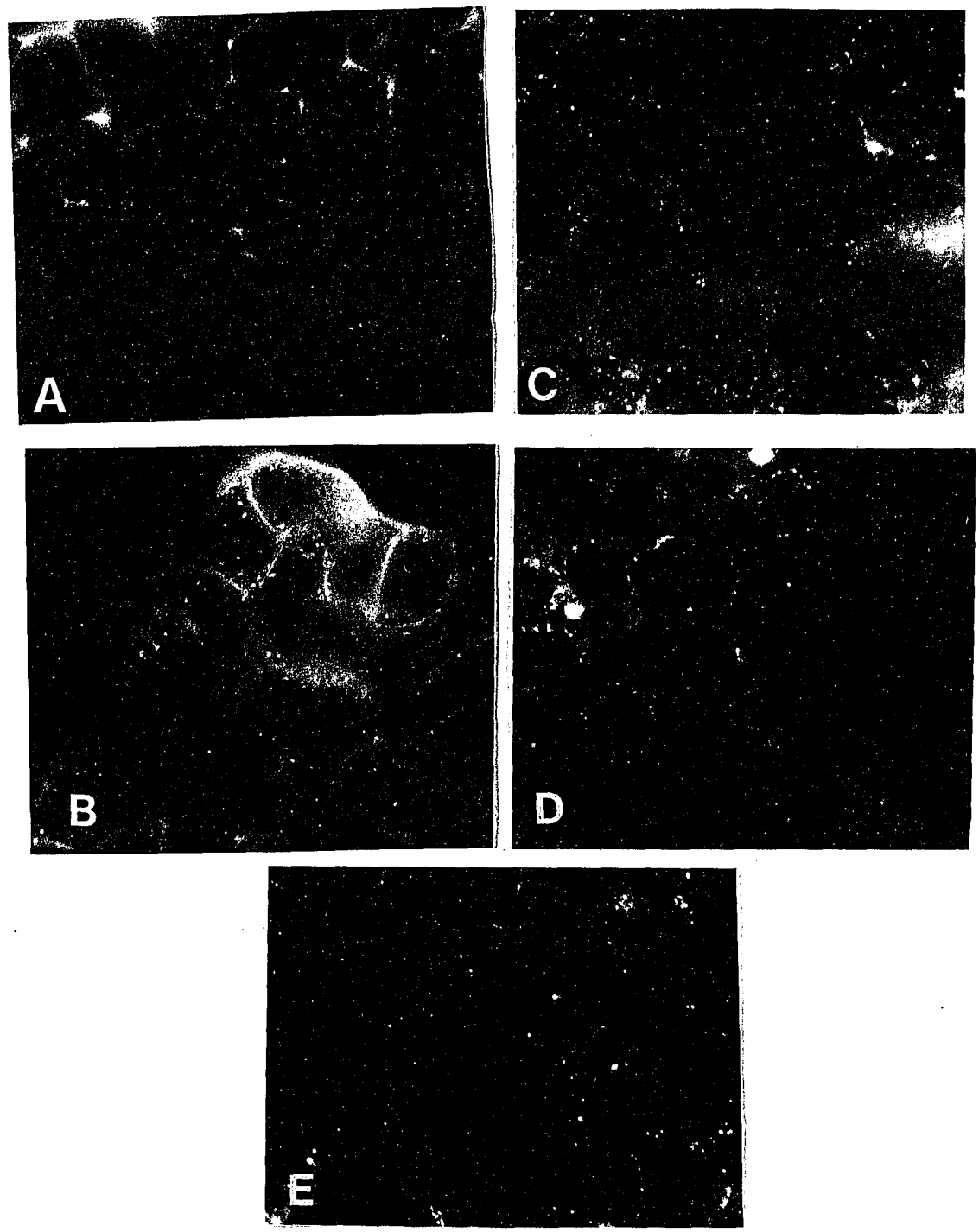

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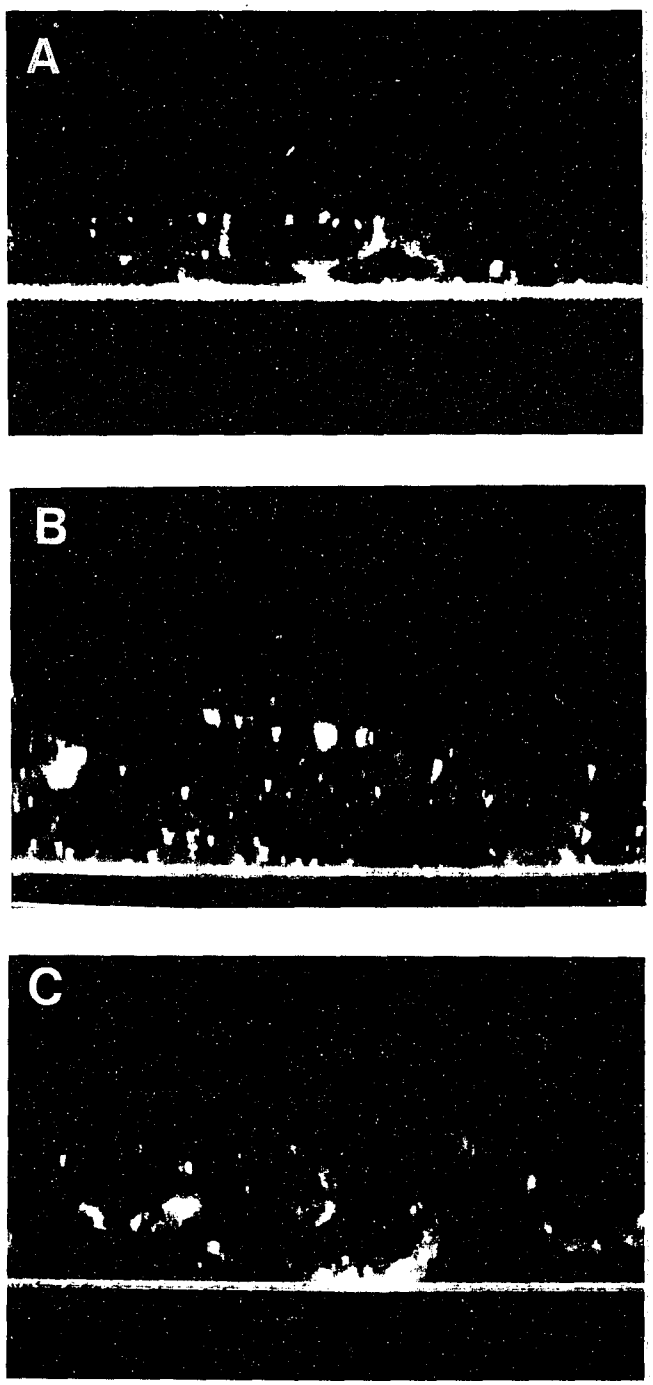

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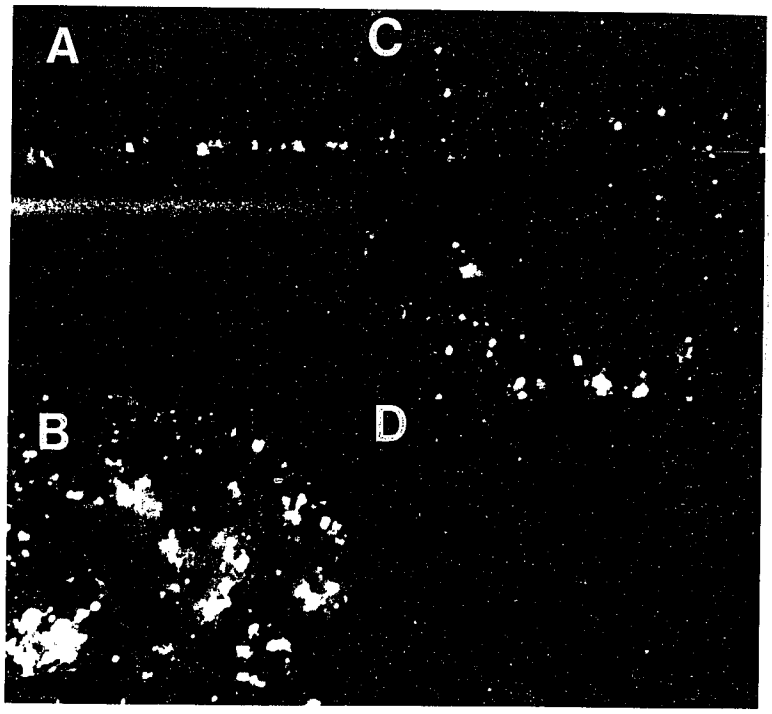

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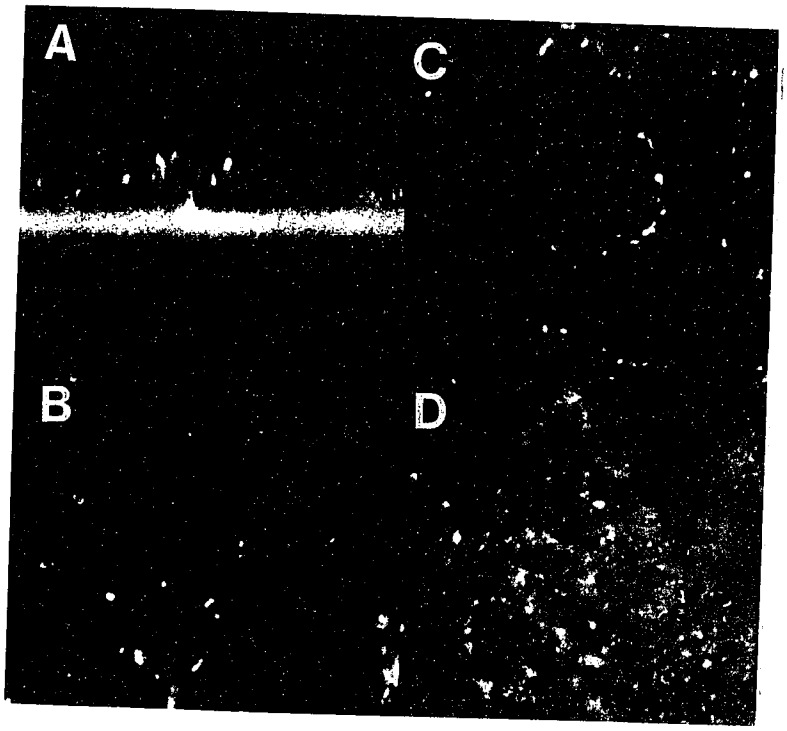

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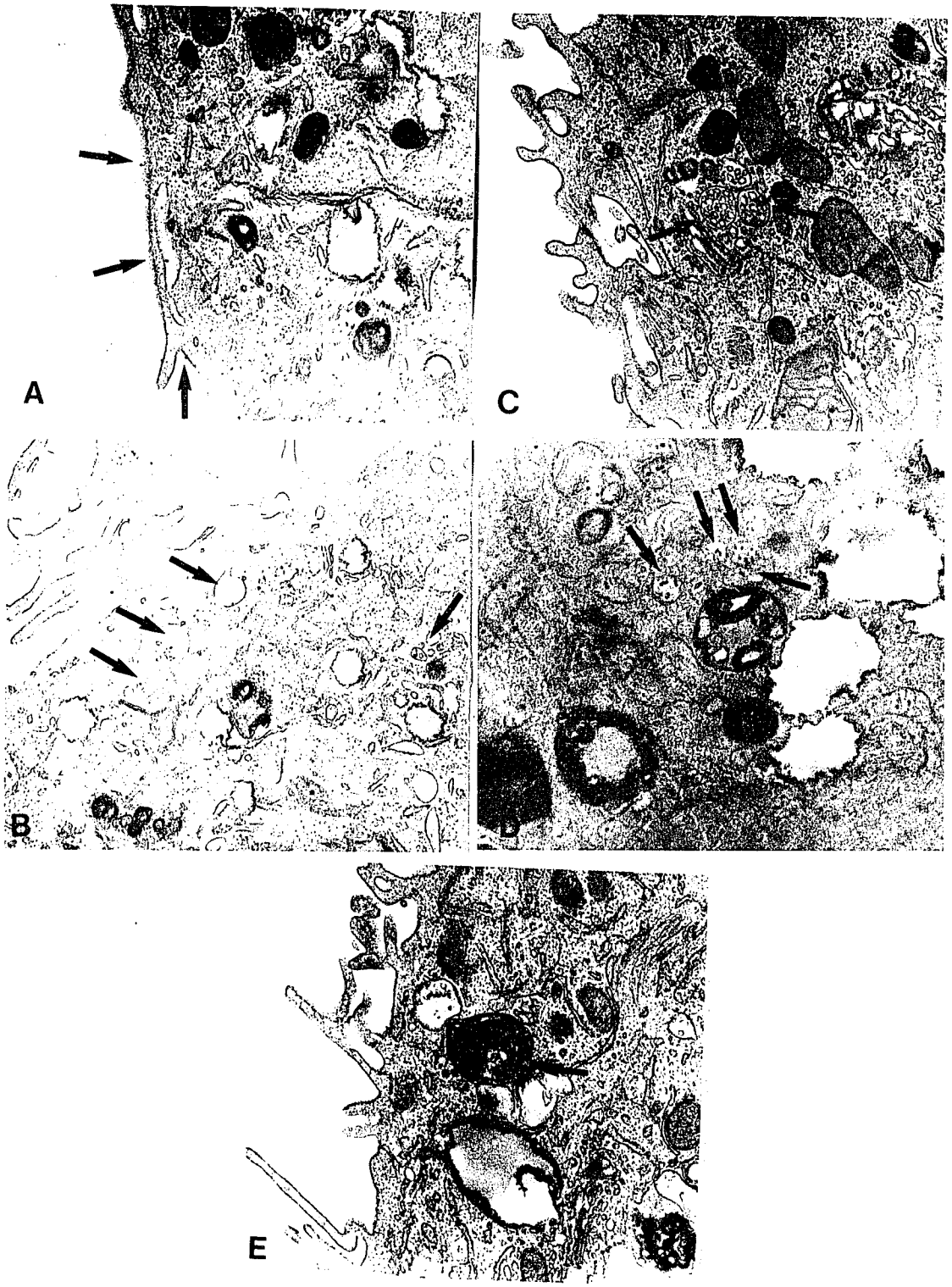

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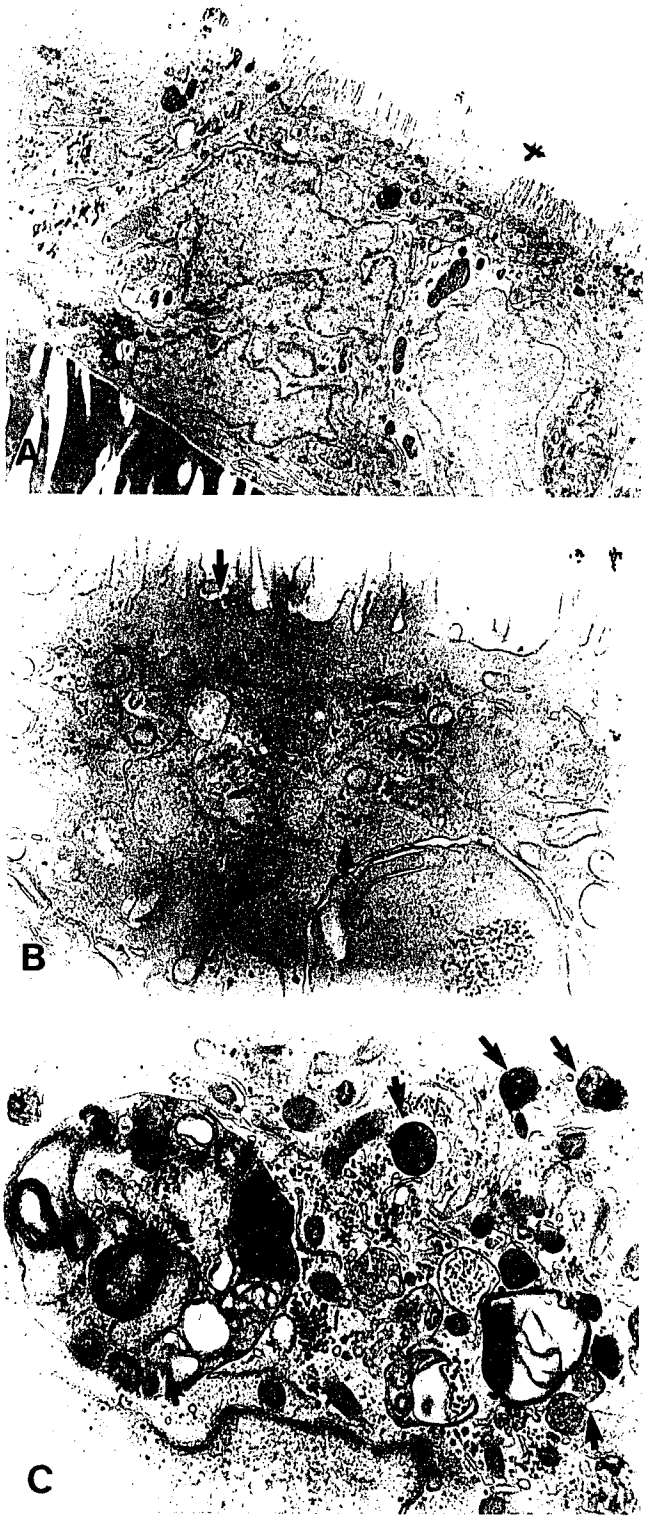

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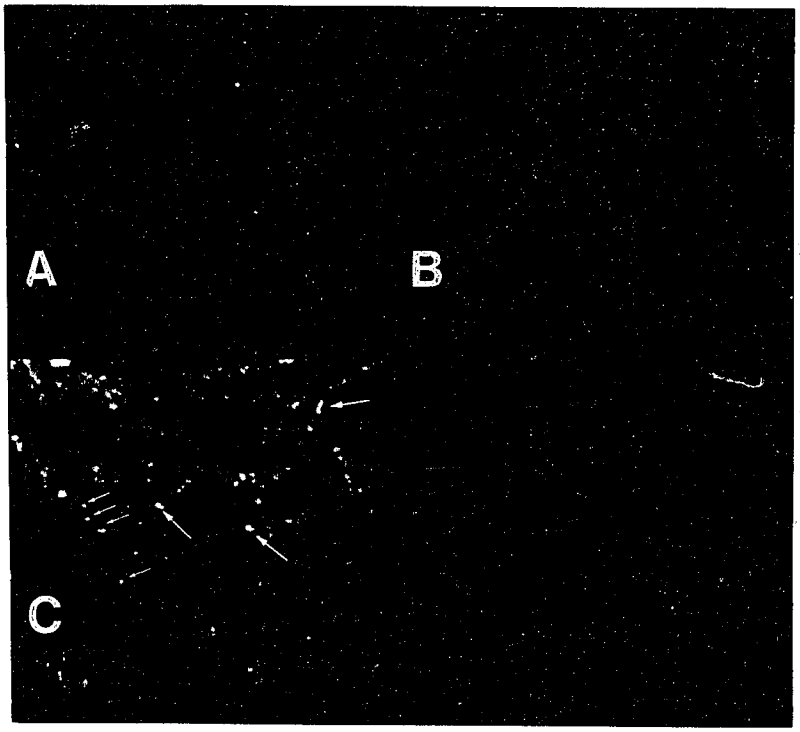

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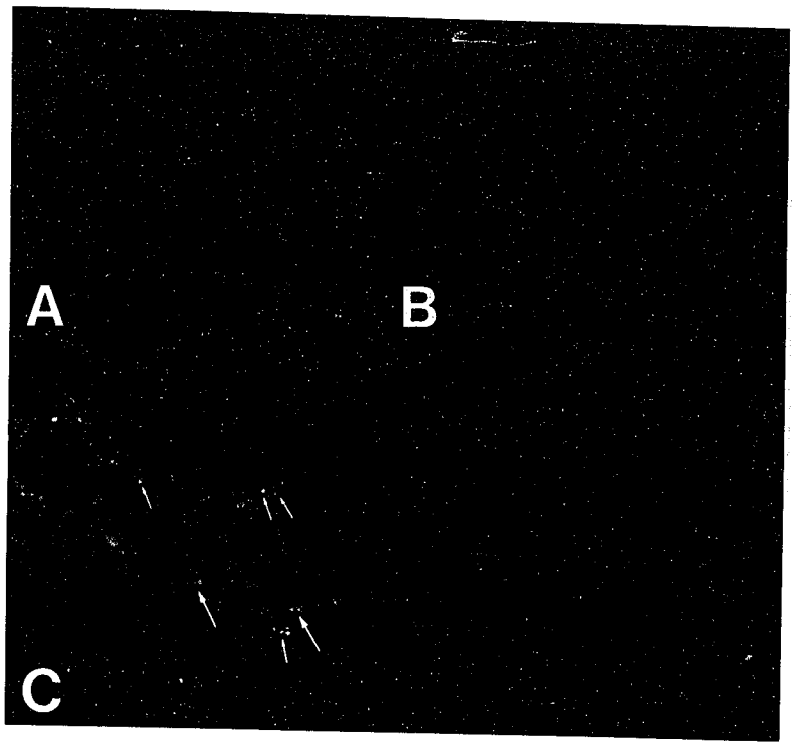

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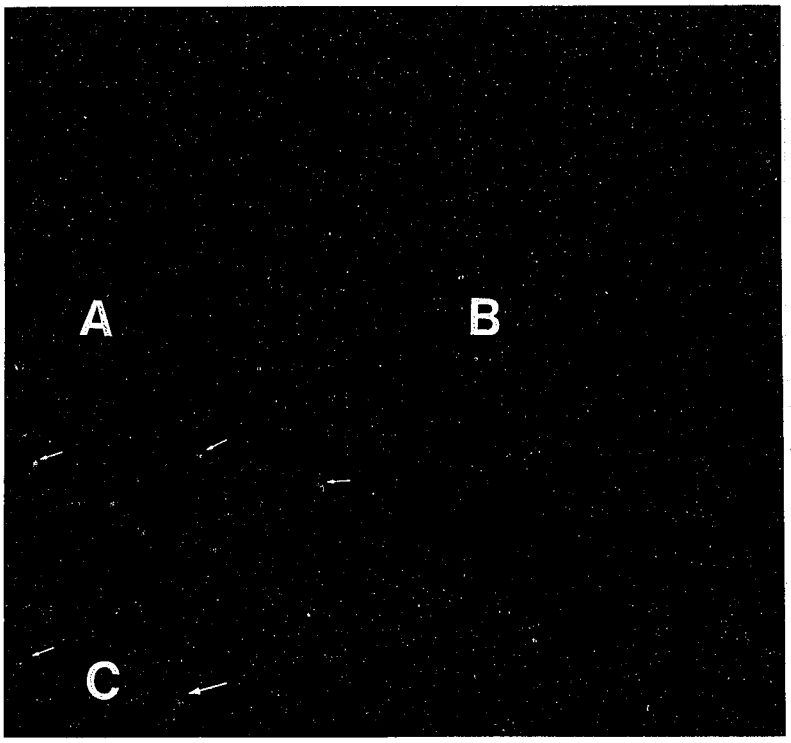

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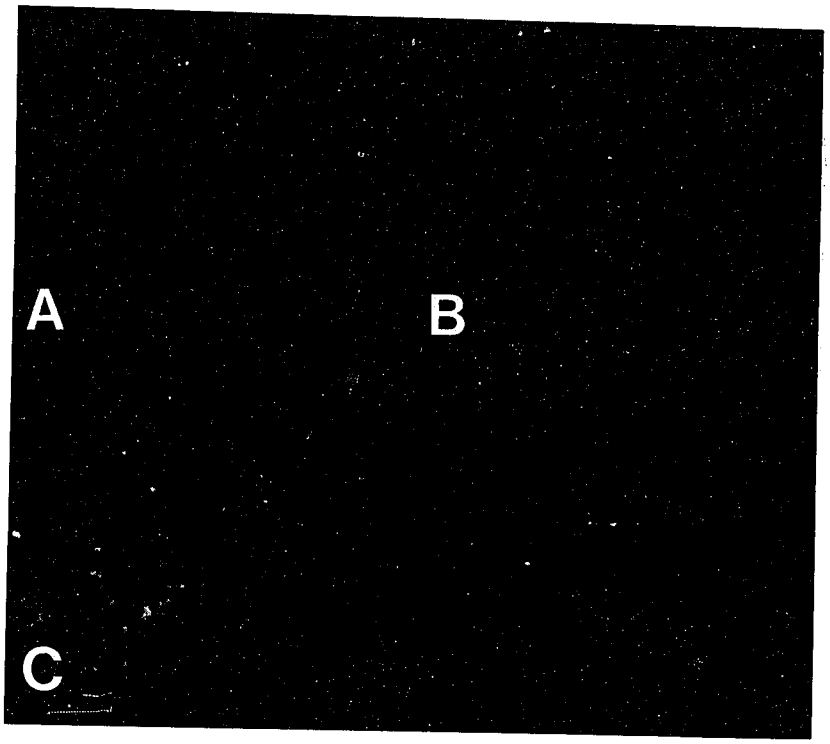

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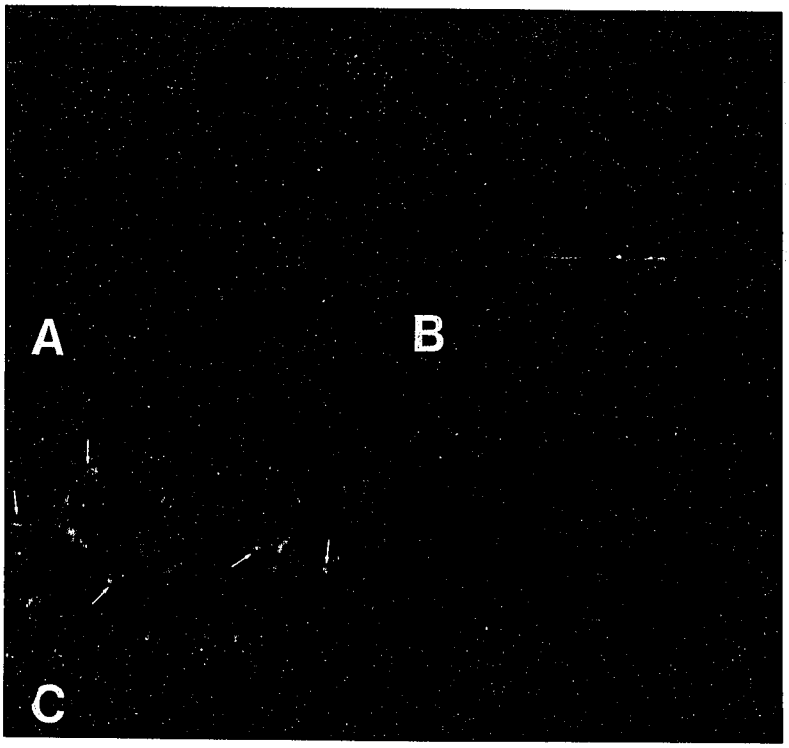

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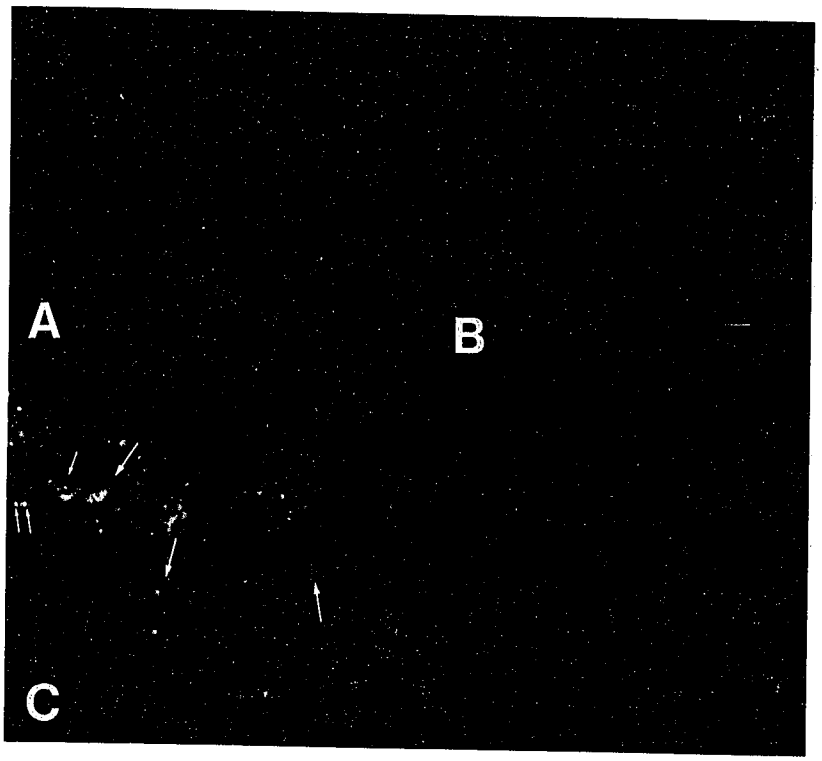

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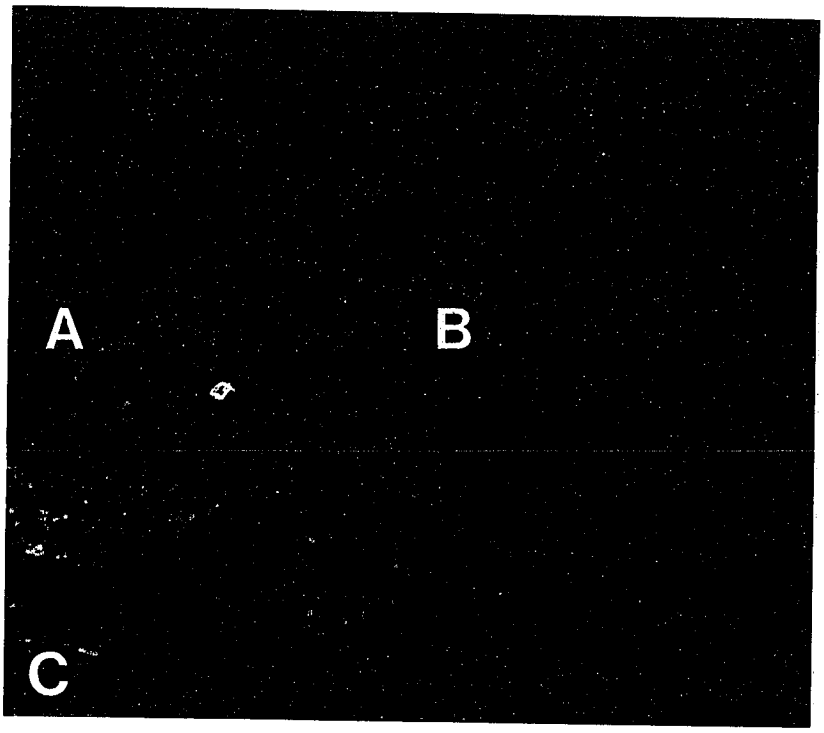

Figure 15

Complementary Results

Kinetics of Antigen Uptake By Caco-2 cells determined by fluorescence microscopy

Caco-2 cells grown on glass coverslips were pulsed with 60 μg of FITC-TT (2 mg/ml) and incubated at 37°C for varying periods, after which they were washed in PBS, fixed with methanol:acetic acid (3:1), and examined by fluorescence microscopy. As shown in Fig. 1, the uptake of FITC-TT was similar to HT29 cells where punctate fluorescence signals inside the cells were not evident until 30 min incubation at 37°C (Fig. 1B). Therefore, the uptake of TT by Caco-2 was also slower than that of monocytes. In contrast to monocytes, no diminution of fluorescence signals was observed in Caco-2 even after 3 h incubation (Fig. 1D), suggesting that either the recycling of endocytic vesicles or the processing of TT by IEC is slower compared to monocytes. A series of pulse-chase experiments were performed in which Caco-2 cells were pulsed with FITC-TT for 60 min at 37°C and chased at 37°C for varying periods. Similar to the findings in HT29, no significant diminution of signals was observed (data not shown). These results concur with the findings observed in HT29 cells.

Kinetics of Antigen Uptake By Caco-2 cells assessed by subcellular fractionation

To confirm the data that we observed in the uptake of fluorescein or gold labeled antigen, we employed subcellular fractionation to isolate endosomal vesicles and examined the uptake of uncoupled TT in Caco-2 cells. Caco-2 cells were incubated with TT for varying periods at 37°C, washed in sucrose buffer, disrupted by a nitrogen bomb, and processed for subcellular fractionation. Samples from the homogenate, low speed pellet (comprised of unbroken cells, nuclei, mitochondria etc.), supernatant, and high speed crude membrane pellet (composed mostly of endocytic vesicles, lysosomes, peroxisomes etc.) fractions were separated on a 10% SDS polyacrylamide gel and transblotted onto a nitrocellulose membrane. Western blot was then performed to detect TT by a rabbit anti-TT antibody. As shown in Fig. 2, an insignificant amount of TT was detected in the homogenate, supernatant, and crude membrane fractions at 15 min. The amount of TT in the crude membrane fractions increased with time, indicating that more TT was internalized into the endocytic vesicles. Consistent with a previous report on the processing of ovalbumin by isolated rat IEC (Bland and Whiting, 1989), limited processing of TT was also observed, suggesting that IEC are less efficient in antigen processing compared with conventional APC.

Antigen processing by HT29 cells

To better evaluate the kinetics and efficiency of antigen processing, we incubated either HT29 cells or monocytes with ^{125}I-OVA for varying periods at 37°C. The cells were then lysed and the nuclei and supernatants (debris free) were obtained for TCA precipitation to determine the percentage of intact ^{125}I-OVA radioactivity. After processing, the amount of TCA precipitable ^{125}I-OVA should reduce. As seen in Fig. 3, the percentage of intact ^{125}I-OVA radioactivity in the supernatant did not significantly change in HT29 cells with time (Fig. 3). In contrast, the amount of intact ^{125}I-OVA decreased markedly with time in monocytes. Thus, HT29 cells are less efficient in antigen processing than monocytes.

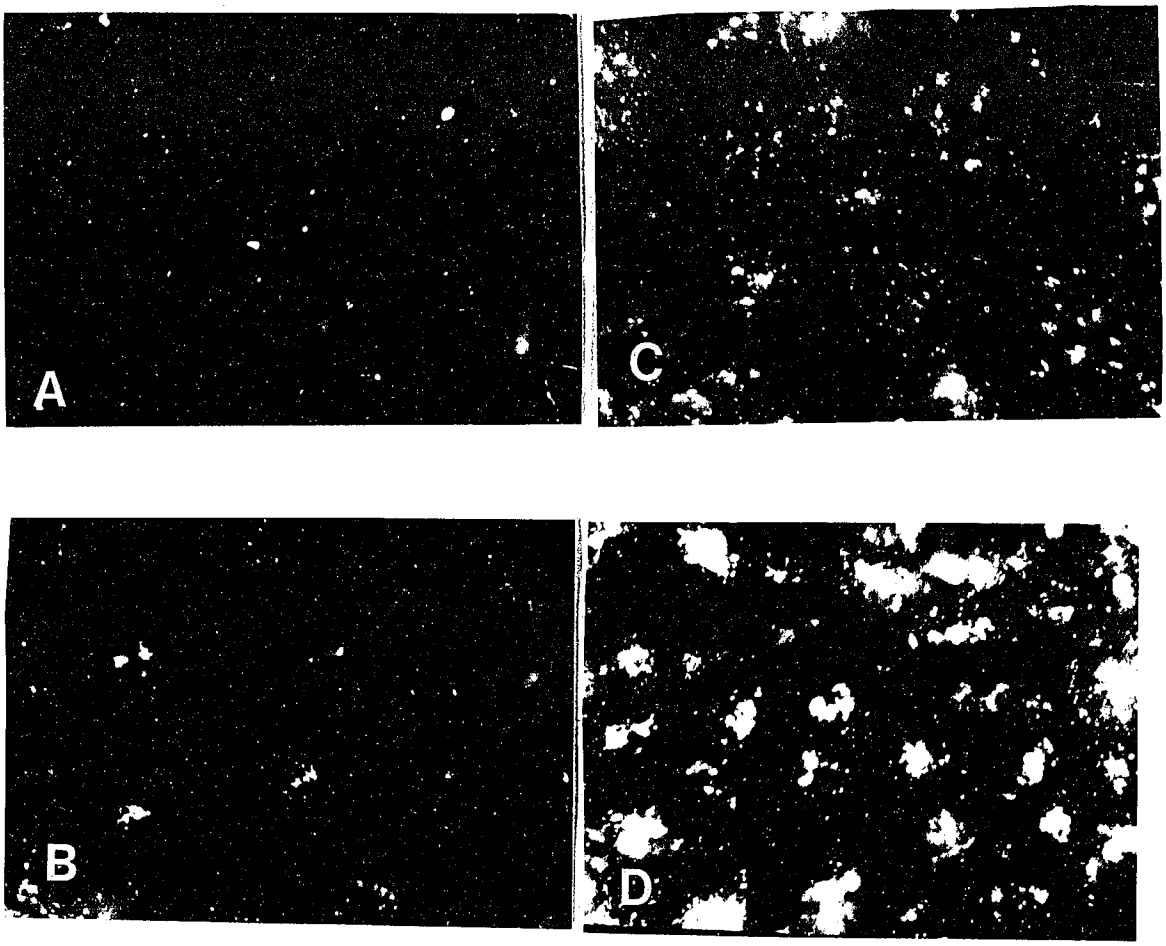

Figure 1. Kinetic studies of FITC-TT uptake by Caco-2. Caco-2 cells grown on coverslips were incubated with 60 μg of FITC-TT for 15 min (A), 30 min (B), 60 min (C), and 3h (D) at 37°C prior to fixation with methanol:acetic acid (3:1). Intracellular fluorescence staining was evident at 30 min and no significant diminution of signal was noted by 3 h. (X100.)

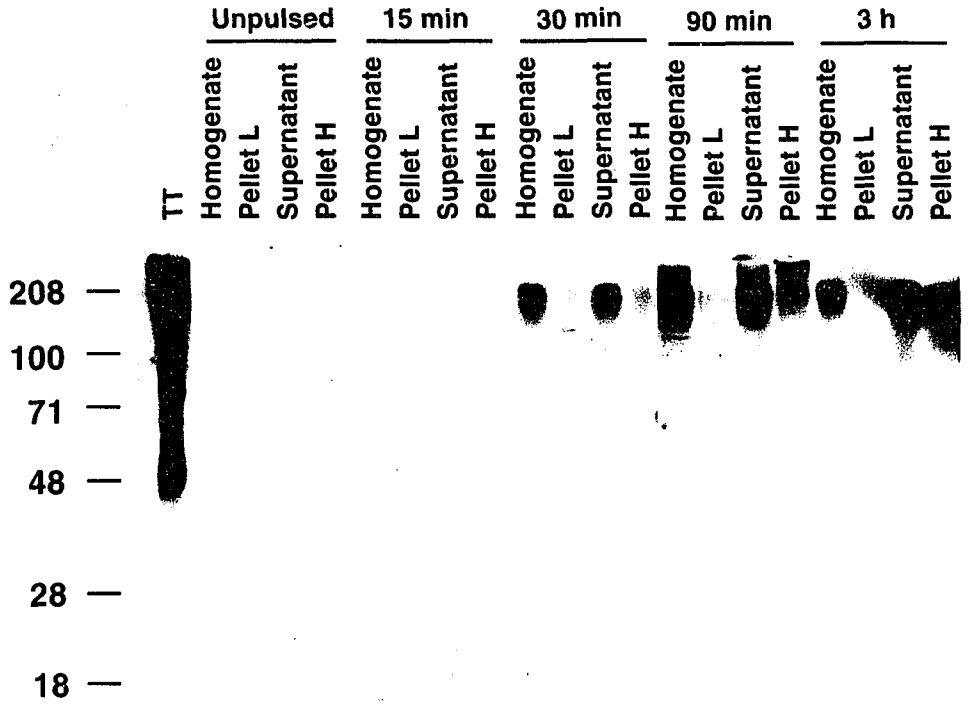

Figure 2. Kinetic studies of TT uptake by Caco-2. Caco-2 cells were incubated with TT for varying periods at 37°C, washed in sucrose buffer, disrupted by a nitrogen bomb, and processed for subcellular fractionation. Samples from the homogenate, low speed pellet (pellet L), supernatant, and crude membrane pellet (pellet H) fractions were separated on a 10% SDS polyacrylamide gel and transblotted onto a nitrocellulose membrane. Western blot was then performed to detect TT with a rabbit anti-TT antibody. The amount of TT detected in the crude membrane fractions (pellet H) increased with time.

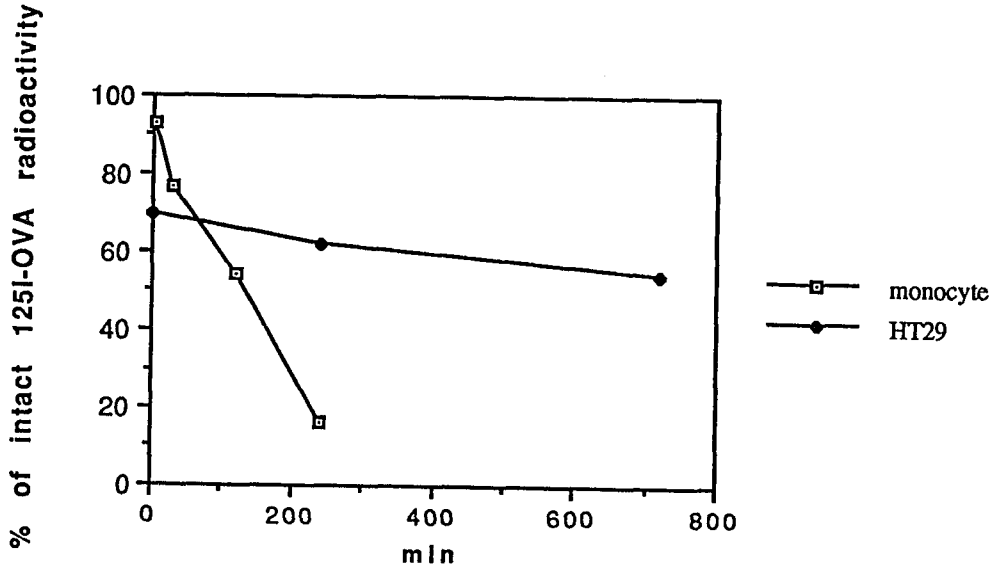

Figure 3. Catabolism of ^{125}I-OVA by HT29 and monocytes. HT29 cells or monocytes were incubated with ^{125}I-OVA for varying periods at 37^{o}C. The cells were then lysed and the supernatants were isolated for TCA precipitation to determine the percentage of radioactivity in intact ^{125}I-OVA. Limited processing of ^{125}I-OVA was evident in HT29, whereas antigen processing was quite rapid in monocytes.

FACTORS AFFECTING ANTIGEN UPTAKE BY HUMAN INTESTINAL EPITHELIAL CELL LINES

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Running title: Antigen Uptake by Intestinal Epithelium

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Abbreviations used in this paper:
FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN-γ, interferon-gamma; Ig, immunoglobulin; KLH; keyhole limpet hemocyanin; MHC, major histocompatibility complex; OVA, ovalbumin; pIgR, polymeric immunoglobulin receptor; TT, tetanus toxoid.

ABSTRACT

Our laboratory has previously shown that human intestinal epithelial cell lines internalize soluble protein antigens with kinetics slower than that of monocytes. To further explore antigen uptake in these unconventional antigen presenting cells, we employed confocal microscopy to examine factors which might affect this process. We assessed the role of size in antigen uptake and kinetics using keyhole limpet hemocyanin and ovalbumin. Both fluoresceinated-keyhole limpet hemocyanin (3,000 - 7,500 KD) and fluoresceinated-ovalbumin (45 KD) were internalized by HT29, a human colonic cell line, with kinetics similar to those of fluoresceinated-tetanus toxoid. We next complexed fluoresceinated-tetanus toxoid with either IgG anti-tetanus or IgG F(ab)'2 anti-tetanus to assess the role of immune complexes in antigen uptake by HT29. In contrast to monocytes where insoluble anti-tetanus immune complexes could be internalized and soluble immune complexes enhanced the uptake of tetanus toxoid, in HT29 decreased uptake of insoluble immune complexes was observed and soluble immune complexes did not enhance uptake. There was also a decrease in the uptake of heat-aggregated tetanus toxoid by HT29 confirming that intestinal epithelial cells are poorly phagocytic cells. Lastly, since intestinal epithelial cells are bathed in cytokines that are constitutively secreted by the gut-associated lymphoid tissue, we cultured HT29 with prophagocytic cytokines (e.g. gamma-interferon, granulocyte-monocyte colony-stimulating factor) prior to pulsing with fluoresceinated-tetanus toxoid. Although such cytokines enhance

antigen uptake in monocytes, they neither altered the kinetics of uptake nor enhanced antigen internalization in HT29. Taken together, these data suggest that regardless of the size of soluble antigen, the presence of prophagocytic cytokines, or soluble immune complexes, fluid phase endocytosis of antigen by the intestinal epithelial cells appears to be a stable process.

INTRODUCTION

The gastrointestinal (GI) tract encounters a wide variety of antigens daily. To maintain homeostasis in the body, the intestinal epithelium works hand in hand with the gut-associated lymphoid tissue to regulate gut mucosal immune responses. The intestinal epithelium is not just a passive physical barrier serving to exclude the entry of antigens. Proteins derived from dietary sources are able to cross the intestinal epithelium to allow the GI tract to fulfill its primary role in nutrient absorption. However, digestion is not an absolute prerequisite for antigen uptake by the intestinal epithelium. A small amount of dietary proteins can escape preprocessing in the lumen and enter the systemic circulation (1). In fact, limited intact antigen entry through the intestinal epithelium might allow for the interaction between antigens and immunocompetent cells of the mucosa to elicit antigen-specific mucosal immune responses which are important for immunosurveillance in the gut. Intact macromolecules like ferritin and horseradish peroxidase are known to be taken up by the intestinal epithelial cells (2, 3). Our laboratory has also shown that colonic epithelial cell lines (HT29 and Caco-2) can take up soluble proteins such as tetanus toxoid (TT) and transferrin. Besides their physiological function as nutrient absorptive cells, the intestinal epithelial cells have been shown to act as unconventional antigen presenting cells in the gut mucosal immune system. Similar to the conventional antigen presenting cells (e.g. macrophages, B cells), the intestinal epithelial cells express major histocompatibility complex

(MHC) class II molecules constitutively (4, 5). Most importantly, they can process and present soluble antigens to stimulate autologous T cells in vitro (6-8). Interestingly, T cells which are activated by the intestinal epithelial cells are predominantly $CD8^+$ T suppressor cells. Since the immune response within the gut appears to be distinct from that of the systemic immune system, the manner by which the intestinal epithelial cells deal with antigen uptake might differ from that of conventional antigen presenting cells. In this study, we examine the factors which might affect antigen uptake by the intestinal epithelial cell lines and compare those with monocytes. Solubility of antigens appears to be a critical factor in antigen uptake by the intestinal epithelial cells. Insoluble antigens are excluded from the uptake while neither the size of antigen nor the presence of prophagocytic cytokines alter the quantity or kinetics of this process.

METHODS AND MATERIALS

CELLS

HT29, purchased from the American Type Culture Collection, was maintained at 37°C, 5% CO_2/95% air in RPMI 1640 (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (Gibco), 1% penicillin/streptomycin (ICN Biomedical, Irvine, CA), and 2mM glutamine (ICN Biomedical). The Caco-2 cell line, a gift from Dr. M. W. Musch (University of Chicago), was maintained under similar conditions in DMEM (Gibco) containing 25 mM glucose (Sigma Chemical Co., St. Louis, MO), 20% heat-inactivated fetal calf serum with both essential and non-essential amino acids (Gibco Laboratories), 10 mM HEPES pH 7.4 (Sigma), and 1% penicillin/streptomycin. HT29 and Caco-2 cells were split 1:3 by 1X trypsin/EDTA (Sigma, St. Louis, MO) once a week and cultured in T175 flasks (Nunc, Naperville, IL).

Peripheral blood mononuclear cells were obtained by Ficoll/Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation. Mononuclear cells were collected from the interface and washed three times in PBS. The cells were resuspended in RPMI 1640 and the cell density was adjusted to $5x10^6$/ml. Monocytes were isolated by plastic adherence by plating peripheral mononuclear cells in tissue culture dishes (Falcon, Lincoln, NJ). Adherent monocytes were removed by incubation at 4°C for 1 h followed by vortexing.

LABELING ANTIGEN WITH FLUORESCEIN

Antigen (e.g. TT, OVA, KLH, IgG) was dialyzed against conjugation buffer (carbonate buffer, pH 10.0) at 4°C with two changes of buffer. 500 μg of fluorescein isothiocyanate (FITC; 25 mg/ml) was added to 1 ml of dialyzed soluble antigen (5-7 mg/ml) and the mixture was rotated overnight in the dark at 4°C. Unbound FITC was removed by passing the mixture through a sephadex G-25 column (Pharmacia). Fractions were collected and O.D.s at 280 nm and 495 nm were measured by a Spectronic 601 spectrophotometer. Fractions with $O.D._{.280}/O.D._{.485}$ greater than 0.8 were used and the final protein concentration was adjusted to 2 mg/ml prior to pulsing.

CYTOKINE PRETREATMENT

Intestinal epithelial cells grown on glass coverslips were treated with 100 U/ml gamma-interferon (IFN-γ; Boehringer Mannheim, Indianapolis, IN) for 48 h or 25 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF; R & D Systems, Minneapolis, MN) for 72 h before pulsing with the FITC-labeled antigen. Similarly, monocytes were pretreated with 100 U/ml IFN-γ or 25 ng/ml GM-CSF for 24-48 h. These concentrations were chosen as optimal for enhancing antigen uptake by monocytes. Antigen uptake was quantitated by measuring fluorescence intensity on a per cell basis with the confocal microscope (see below).

FORMATION OF IMMUNE COMPLEXES AND HEAT-AGGREGATED TT

25 mg/ml of human anti-TT IgG (Miles Inc., Elkhart, IN) was incubated with 8 mg/ml of FITC-TT at 37°C for 1 h. The insoluble

immune complexes were separated from the soluble immune complexes by centrifuging at 10,000g for 1h. Before using in the pulsing studies, insoluble immune complexes were resuspended in RPMI and the concentration was adjusted to 2 mg/ml. To prepare anti-TT IgG F(ab)'$_2$ fragments, anti-TT IgG antibody was dialyzed against 0.1M sodium acetate buffer, pH4.5 overnight and then digested with 4% pepsin at 37^{o}C overnight. The F(ab)'$_2$ fragments were then purified by a sephadex G150 column. SDS polyacrylamide gel electrophoresis was used to confirm the fractions containing the anti-TT IgG F(ab)'$_2$ fragments. Anti-TT IgG F(ab)'$_2$/FITC-TT immune complexes were formed in the same manner as the immune complexes with intact anti-TT IgG antibody. To form heat-aggregated FITC-TT, FITC-TT was heated at 68^{o}C for 1 h. The heat-aggregated FITC-TT was isolated by centrifuging at 10,000g for 1 h. Before pulsing, heat-aggregated FITC-TT was resuspended in RPMI and the concentration was adjusted to 2 mg/ml.

LASER SCANNING CONFOCAL MICROSCOPY

Intestinal epithelial cells were incubated with 60 μg of FITC-TT at 37^{o}C for varying periods. After incubation, the cells were washed in PBS three times, and fixed with methanol:acetic acid (3:1) at -20^{o}C for 5 min. The cells were further washed in PBS before mounting with Immu-mount (Shandon). Monocytes were treated in the same manner except that they were cytospun (1000 rpm for 10 min by cytospin, Shandon) onto glass slides before fixation. The slides were then viewed by a Leica fluovert laser scanning confocal microscope at a step position of 1 μm on the X-Y axis using the

accompanying software. The intensity of fluorescence signals were measured with constant parameters (e.g. pinhole, voltage, offset) in the same set of experiments. Images were photographed from the computer monitor with Kodak Ektachrome 100Hc or T-Max 100 film.

RESULTS

EFFECT OF SIZE OF ANTIGEN ON UPTAKE BY HT29

In addition to TT, intestinal epithelial cells have been reported to present soluble antigens such as keyhole limpet hemocyanin (KLH) (8) or ovalbumin (OVA) (6) to MHC restricted T cells. KLH is a heterogeneous molecule with molecular weights varying from 3,000 to 7,500 KD, whereas OVA is only 45 KD. We conjugated these antigens with FITC to determine the effect of size on the kinetics of antigen uptake. Regardless of size, both FITC-KLH (figure 1) and FITC-OVA (figure 2) were taken up by HT29 with kinetics similar to those seen with FITC-TT. In both cases, fluoresceinated antigens were taken up into cellular compartments and appeared as punctate staining at 30 min. Similar results were observed in Caco-2 cells (data not shown). Thus, the rate of fluid phase endocytosis in HT29 is not affected by the size of soluble protein antigens.

EFFECT OF IMMUNE COMPLEXES ON ANTIGEN UPTAKE BY HT29

sIgA and sIgM in the mucosal secretions and gut lumen have been clearly defined to play a vital role in limiting antigen entry through the intestinal epithelium by preventing the binding of antigens to the intestinal epithelium. In IgA deficient patients, it has been shown that IgG and IgM can compensate for the antiviral activity by neutralizing the virus in the external mucosal surfaces (9).

However, little is known about the effect of IgG immune complexes on antigen uptake by the intestinal epithelial cells. Previous studies have documented the existence of IgG Fc receptors on neonatal rat intestinal epithelial cells (10) and human goblet cells (11). Since Fc receptors play an important role in antigen uptake by macrophages and there is an increasing appreciation of the fact that IgG can get into the secretions from the serum by passive diffusion, fluid phase endocytosis, or in some cases via the formation of sIgA-IgG complexes, we therefore studied the effect of complexed FITC-TT with IgG anti-TT as soluble and insoluble immune complexes on the uptake by HT29. The optimum concentrations of FITC-TT used to form insoluble immune complexes with IgG anti-TT and IgG F(ab)'2 anti-TT were determined by performing Ouchterlony or nephelometric analysis. Insoluble immune complexes were resuspended in RPMI before pulsing. Compared with soluble FITC-TT, there was a decrease of uptake of insoluble anti-TT immune complexes (figure 3). To ensure that the Fc portion of the IgG was not playing a role in this process, we co-cultured IgG F(ab)'2 anti-TT (figure 4) immune complexes with HT29. Similar to the intact antibodies, there was also a reduction in uptake. To exclude the possibility that the anti-TT antibody itself was mediating the effect, the uptake of FITC-IgG anti-TT alone without antigen was also assessed. Similar to other soluble protein antigens, FITC-IgG anti-TT was also taken up by HT29 with kinetics similar to TT (figure 5). These findings suggest that antigen itself is not a factor but solubility of antigen could play a role. To test this more directly, the uptake of heat-aggregated FITC-TT was also examined.

As seen above for other insoluble proteins, there was a decrease in antigen uptake (figure 6). This reduction in uptake appears to relate to the physical form of the antigen. Insoluble intact IgG or F(ab)'2 anti-TT immune complexes and heat-aggregated FITC-TT also caused a reduction of uptake in Caco-2 cells (not shown). Insoluble immune complexes are poorly endocytosed when compared with the controls. In contrast, monocytes were capable of taking up insoluble immune complexes as well as heat-aggregated TT (figure 7). Interestingly, soluble immune complexes did not enhance the uptake of FITC-TT although they did in monocytes (figure 8). This is not surprising since HT29 cells fail to express Fc receptors (data not shown). These data suggest that insoluble immune complexes formed in the lumen are excluded from the intestinal epithelium and only soluble antigens can be sampled by the intestinal epithelial cells.

EFFECT OF PROPHAGOCYTIC CYTOKINES ON ANTIGEN UPTAKE BY HT29

Numerous cytokines have been shown to regulate antigen uptake by conventional antigen presenting cells. Since our laboratory has shown that freshly isolated epithelial cells and HT29 cells express receptors for IFN-γ and GM-CSF (12), conceivably these cytokines might enhance antigen uptake in intestinal epithelial cells as they do in monocytes. To examine the effect of prophagocytic cytokines on HT29 cells, HT29 cells grown on coverslips were pretreated with 100 U/ml of IFN-γ for 48 h or 25 ng/ml of GM-CSF for 72 h before

the antigen uptake assay. HT29 cells were then incubated with FITC-TT at 37°C for varying periods. The kinetics of antigen uptake in IFN-γ (figure 9) or GM-CSF (figure 10) pretreated HT29 cells was similar to untreated cells. Punctate staining was evident only at 30 min in either scenario. Although the kinetics of antigen uptake was unchanged by either cytokine, it was possible that the endocytic capacity might be enhanced in the cells. In monocytes, both IFN-γ and GM-CSF pretreatments enhanced the endocytosis of antigen (figure 11). The amount of antigen taken up on a per cell basis as a reflection of endocytic capacity was measured by confocal microscopy. Individual cells were localized after pulsing and total fluorescence intensity per cell was measured. The results shown in figure 12 are the conglomerate of several experiments. There was a 2-fold increase in FITC-TT uptake in monocytes pretreated with either IFN-γ or GM-CSF. In contrast, treatment with prophagocytic cytokines did not seem to enhance antigen uptake in HT29 cells. Varying the incubation period of IFN-γ or GM-CSF with HT29 from 24 to 72 h did not alter the results. Neither IFN-γ nor GM-CSF had any effect on the kinetics or quantity of antigen uptake in Caco-2 cells (data not shown). Hence, these prophagocytic cytokines do not alter the kinetics or amount of antigen uptake in the intestinal epithelial cell lines.

DISCUSSION

The data presented here suggest that antigen uptake in the intestinal epithelial cell lines is a stable process although the physical form (soluble vs. insoluble) of antigen appears to play a role. Several differences were noted between monocytes and intestinal epithelial cells. Prophagocytic cytokines which usually augment antigen uptake in monocytes had no significant effect on antigen uptake in the intestinal epithelial cell lines. Furthermore, IgG immune complexes were handled differently by the monocytes and intestinal epithelial cells. Opsonization promoted antigen uptake in monocytes, whereas insoluble IgG immune complexes impeded antigen uptake by the intestinal epithelial cell lines. Thus, the regulation of antigen uptake in the intestinal epithelial cell lines is very different from that of conventional antigen presenting cells such as monocytes.

Our data show that as long as protein antigens are in soluble form, they can be taken up by the intestinal epithelial cells. The nonspecific fluid phase endocytosis by the intestinal epithelial cells appears to be a slow and stable process. Unlike passive diffusion, the size of antigens does not affect the rate of endocytosis. These data suggest that the uptake of soluble protein antigen via fluid phase endocytosis by the intestinal epithelial cells is tightly regulated. There is no correlation between the kinetics of antigen uptake and the size of antigen.

It is known that immunoglobulin is part of the mucosal barrier that excludes the entry of antigen by preventing the binding of antigen to the intestinal epithelium. IgG has also been demonstrated to restrict the replication of virus by neutralizing viruses (13). Moreover, infusion of immune complexes (bovine serum albumin (BSA) with rat serum anti-BSA antibodies) into the rat duodenum has been shown to stimulate goblet cells to secrete more mucus (14). The enhanced mucus secretion serves to trap antigens and prevent antigens gaining access to the underlying epithelium. In this study, we show that immunoglobulin also helps limit antigen uptake by forming insoluble immune complexes. These findings are correlated with previous studies where intestinal epithelial cells are non-phagocytic cells and only take up soluble antigens by fluid phase endocytosis. The exception to these findings may be some enterobacteria (e.g. *Yersinia, Salmonella*). *Yersinia or Salmonella* express membrane proteins (e.g. invasin in *Yersinia*) which aid in the attachment of the bacteria to the surface of epithelial cells, triggering a change in the cytoskeleton to facilitate the phagocytosis of the bacteria (15, 16). The exact mechanism as to how these surface proteins trigger bacterial internalization is not known, but receptors for invasin, an integrin-binding protein, are expressed on mammalian epithelial cells and receptor-mediated phagocytosis may play a role (17). Similar scenarios might be applicable to viral infection of the intestinal epithelial cells. Several viral receptors have been identified on the intestinal epithelial cells (18-20). Kaetzel *et al.* have also shown that

polymeric Ig receptors (pIgR) allow for the transcytosis of soluble immune complexes containing dimeric IgA in MDCK cells transfected with pIgR (21). Moreover, Mazanec *et al.* showed that IgA anti-Sendai virus antibody was capable of neutralizing infectivity of Sendai virus intracellularly during vesicular transport (22). However, these are typically from the basolateral to apical surfaces and would not reflect antigen sampling in the lumen. The ability of intestinal epithelial cells to take up insoluble or soluble IgA immune complexes apically has not been determined. In this study, we utilized IgG immune complexes which do not interact with the pIgR on HT29. There is increasing evidence that IgG is present in the lamina propria as well as in the gut lumen and may be increased in certain gastrointestinal diseases (23). Although formation of insoluble IgG immune complexes may help limit the secondary inflammatory response by reducing antigen uptake from the lumen, several studies have shown that excess deposition of IgG immune complexes on the intestinal epithelium and submucosa can activate complement resulting in damage to the intestinal epithelium (24, 25).

Intestinal epithelial cells are in intimate contact with a variety of immunocompetent cells such as lamina propria lymphocytes (LPL), intraepithelial lymphocytes (IEL), and mucosal macrophages which secrete a number of cytokines to regulate the mucosal immune system (26). Intestinal epithelial cells have been shown to express receptors for macrophage activating factors, IFN-γ and GM-CSF (12). IFN-γ is produced by both IEL and LPL (27, 28), whereas GM-CSF is

produced by mucosal macrophages and intestinal epithelial cells (29). In monocytes, both IFN-γ and GM-CSF have been shown to promote antigen presenting functions including the upregulation of the expression of MHC class II molecules, promoting cytokine synthesis, and enhancing phagocytic activity (30, 31). Previous studies have demonstrated that IFN-γ enhances the expression of MHC class II on intestinal epithelial cells in vivo (32) and in vitro (5). Similar observations were made in HT29 where an increase of MHC class II expression was noted after culturing with IFN-γ for 48 h or GM-CSF for 72 h (33). Hence, similar to monocytes, IFN-γ and GM-CSF can enhance the expression of MHC class II molecules on the intestinal epithelial cells. However, despite this finding, these prophagocytic cytokines had no effect on the kinetics or amount of antigen taken up by HT29. Perhaps, the cytoskeletal elements of intestinal epithelial cells are different from those of professional phagocytic cells like monocytes. Therefore, prophagocytic cytokines exert no effect on the endocytic capacity of intestinal epithelial cells. This finding may be quite relevant in terms of controlling antigen entry and sampling through the intestinal epithelium. In many gastrointestinal diseases, there is an increase of cytokine production including IFN-γ and GM-CSF. Our laboratory has recently shown that intestinal epithelial cells derived from patients with inflammatory bowel disease secrete higher levels of GM-CSF than normal controls (29). In addition, IFN-γ, a proinflammatory cytokine, is known to disrupt the tight junctions and increase antigen entry through the paracellular route (34). Therefore, limiting the response of intestinal epithelial cells to conventional prophagocytic

cytokines might prevent further transcytosis of antigen, thus resulting in the failure to provoke further inflammation in the mucosa.

In conclusion, these findings suggest that antigen uptake by the intestinal epithelial cell lines is tightly controlled and distinct from that of monocytes. Thus far, very few factors have been shown to augment antigen uptake by the intestinal epithelial cells. In fact, the insoluble form of antigen actually inhibits uptake. These findings might be beneficial and important with respect to maintaining homeostasis in the GI tract. Selective antigen sampling may aid in antigen exclusion and facilitate directed responses against specific antigens.

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FIGURE LEGENDS

Figure 1. Kinetic studies of FITC-KLH uptake by HT29. HT29 cells grown on coverslips were incubated with 60 μg of FITC-KLH (2 mg/ml) for varying periods at 37°C. The cells were then washed in PBS and fixed with methanol:acetic acid (3:1). No antigen uptake was evident at 15 min (a). Intracellular signals were evident at 30 min (b). Greater fluorescence signals were evident at 60 min (c). (X100.)

Figure 2. Kinetics studies of FITC-OVA uptake by HT29. HT29 cells grown on coverslips were incubated with 60 μg of FITC-OVA (2 mg/ml) for varying periods at 37°C. The cells were then washed in PBS and fixed with methanol:acetic acid (3:1). No antigen uptake was evident at 15 min (a). Intracellular signals were evident at 30 min (b). Greater fluorescence signals were evident at 60 min (c). (X100.)

Figure 3. Uptake of insoluble IgG anti-TT immune complexes by HT29. HT29 cells grown on coverslips were incubated with either 60 μg of insoluble IgG anti-TT immune complexes or 60 μg of FITC-TT for 60 min at 37°C prior to fixation with methanol:acetic acid (3:1). The conglomerate of all sections is shown in a and c, whereas representative cross-sections of HT29 cells are shown in b and d. A decrease of uptake of immune complexes was evident (a, b) when compared with the FITC-TT controls (c, d). (X100.)

Figure 4. Uptake of F(ab)'2 anti-TT immune complexes by HT29. HT29 cells grown on coverslips were incubated with either 60 μg of insoluble F(ab)'2 anti-TT immune complexes or 60 μg of FITC-TT for 60 min at 37°C prior to fixation with methanol:acetic acid (3:1). The conglomerate of all sections is shown in a and c, whereas representative cross-sections of HT29 cells are shown in b and d. A decrease of uptake of immune complexes was evident (a, b) when compared with the FITC-TT controls (c, d). (X100.)

Figure 5. Kinetic studies of FITC-IgG anti-TT uptake by HT29. HT29 cells grown on coverslips were incubated with 60 μg of FITC-IgG anti-TT for 60 min at 37°C prior to fixation with methanol:acetic acid (3:1). No antigen uptake was evident at 15 min (a). Intracellular fluorescence signals were evident at 30 min (b). Increased fluorescence signals were evident at 60 min (c). (X100.)

Figure 6. Uptake of heat-aggregated FITC-TT by HT29. HT29 cells grown on coverslips were incubated with 60 μg of insoluble heat-aggregated TT or 60 μg of FITC-TT for 60 min at 37°C prior to fixation with methanol:acetic acid (3:1). The conglomerate of all sections is shown in a and c, whereas representative cross-sections of HT29 cells are shown in b and d. A decrease of uptake of heat-aggregated FITC-TT uptake was evident (a, b) when compared with the FITC-TT controls (c, d). (X100.)

Figure 7. Uptake of insoluble immune complexes and heat-aggregated FITC-TT by monocytes. Monocytes were incubated with insoluble IgG anti-TT immune complexes, insoluble F(ab)'2 anti-TT immune complexes, as well as insoluble heat-aggregated TT for 10 min prior to fixation with methanol:acetic acid (3:1). Uptake of insoluble IgG anti-TT immune complexes (b), insoluble F(ab)'2 anti-TT immune complexes (c), and heat-aggregated TT (d) were evident. The soluble FITC-TT control is shown in (a). (X100.)

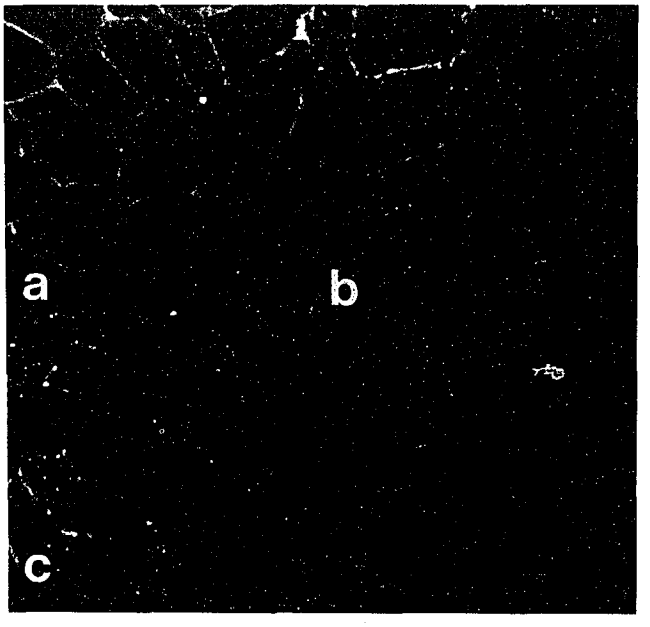
Figure 8. Uptake of soluble IgG anti-TT immune complexes by HT29 and monocytes. HT29 cells grown on coverslips were incubated with 60 μg of soluble IgG anti-TT immune complexes prior to fixation with methanol:acetic acid (3:1), whereas monocytes were incubated with soluble immune complexes for 10 min prior to fixation. Increased uptake of soluble immune complex was evident in monocytes (c) but not in HT29 (a). The soluble FITC-TT controls are shown in (b) and (d). (X100.)

Figure 9. Kinetic studies of FITC-TT uptake by IFN-γ treated HT29. HT29 cells grown on coverslips were treated with 100 U/ml of IFN-γ for 48 h prior to incubation with 60 μg of FITC-TT for varying periods at 37°C. The cells were then washed in PBS and fixed with methanol:acetic acid (3:1). No antigen uptake was evident at 15 min (a). Intracellular signals were evident at 30 min (b). Increased fluorescence signals were evident at 60 min (c). (X100.)

Figure 10. Kinetic studies of FITC-TT uptake by GM-CSF treated HT29. HT29 cells grown on coverslips were treated with 25 ng/ml of GM-CSF for 72 h prior to incubation with 60 μg of FITC-TT for varying periods at 37°C. The cells were then washed in PBS and fixed with methanol:acetic acid (3:1). No antigen uptake was evident at 15 min (a). Intracellular signals were evident at 30 min (b). More punctate staining was evident at 60 min (c). (X100.)

Figure 11. Uptake of FITC-TT by IFN-γ or GM-CSF treated monocytes. Monocytes incubated with IFN-γ (100 U/ml for 48 h), or GM-CSF (25 ng/ml for 48 h) were pulsed with 60 μg of FITC-TT for 10 min at 37°C prior to fixation with methanol:acetic acid (3:1). Increased fluorescence signals were evident in IFN-γ treated (b) as well as GM-CSF treated (c) monocytes when compared with the control (a). (X100.)

Figure 12. Measurement of FITC-TT uptake in HT29 and monocytes. Mean fluorescence intensity was measured by confocal microscopy on a per cell basis. Neither IFN-γ (a) nor GM-CSF (b) enhanced the uptake of FITC-TT, as reflected by the mean fluorescence intensity, in HT29. In contrast, there was a 2-fold increase in FITC-TT uptake in monocytes treated with these cytokines (c). One representative experiment of four.



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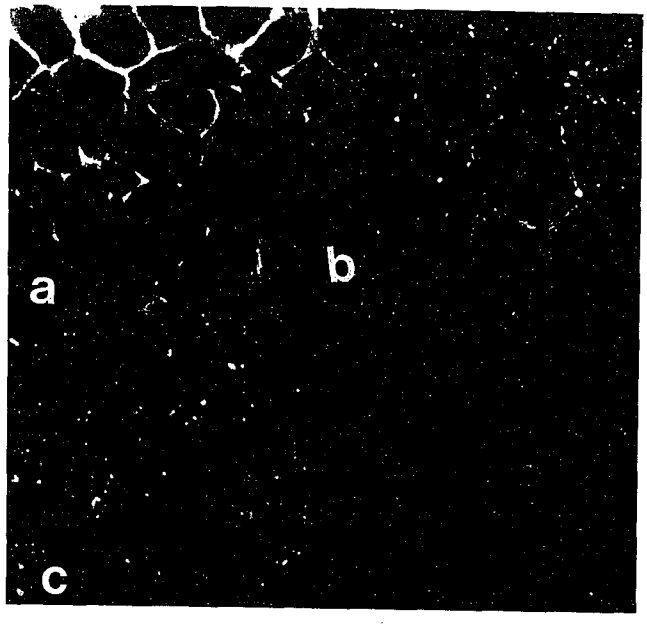

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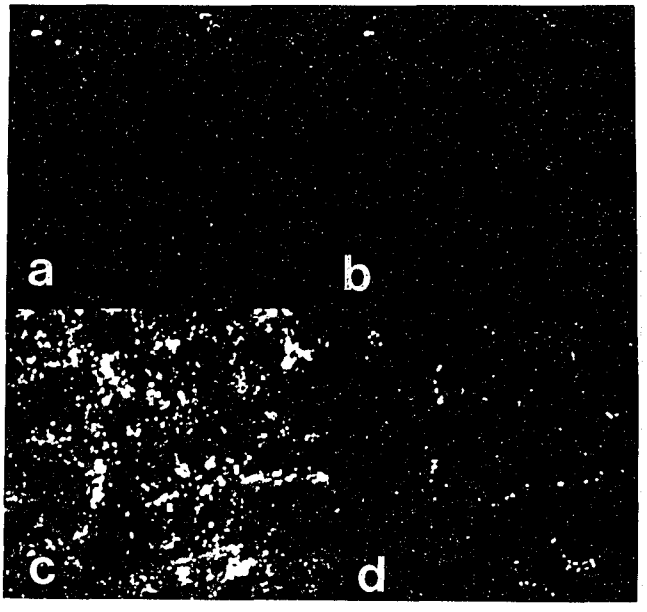

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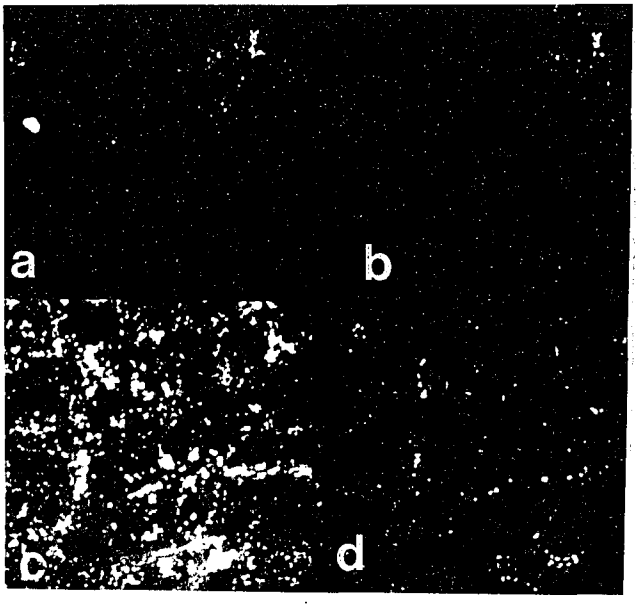


Figure 4. Uptake of F(ab)'2 anti-TT immune complexes by HT29. HT29 cells grown on coverslips were incubated with either 60 μg of insoluble F(ab)'2 anti-TT immune complexes or 60 μg of FITC-TT for 60 min at 37°C prior to fixation with methanol:acetic acid (3:1). The conglomerate of all sections is shown in a and c, whereas representative cross-sections of HT29 cells are shown in b and d. A decrease of uptake of immune complexes was evident (a, b) when compared with the FITC-TT controls (c, d). (X100.)

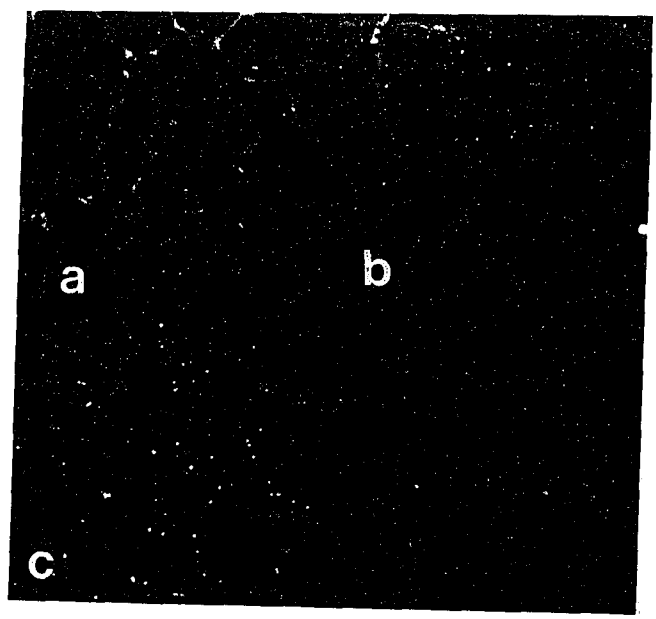


Figure 5. Kinetic studies of FITC-IgG anti-TT uptake by HT29. HT29 cells grown on coverslips were incubated with 60 µg of FITC-IgG anti-TT for 60 min at 37°C prior to fixation with methanol:acetic acid (3:1). No antigen uptake was evident at 15 min (a). Intracellular fluorescence signals were evident at 30 min (b). Increased fluorescence signals were evident at 60 min (c). (X100.)

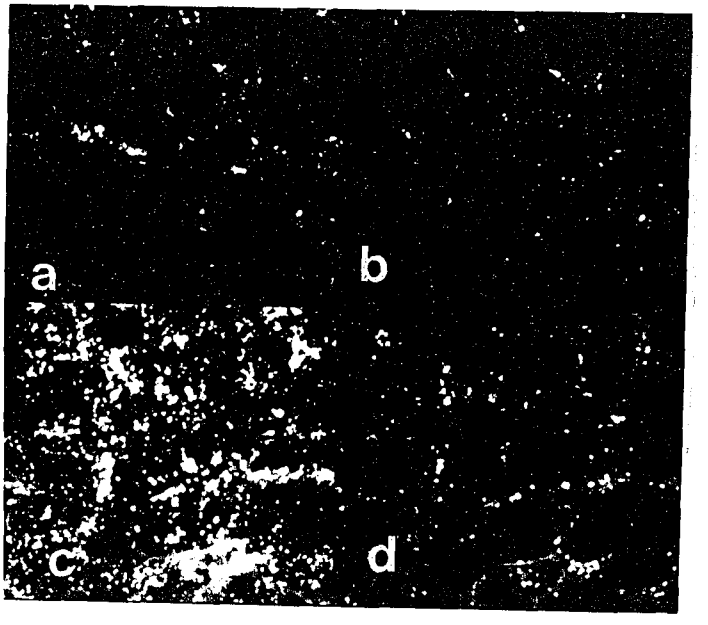
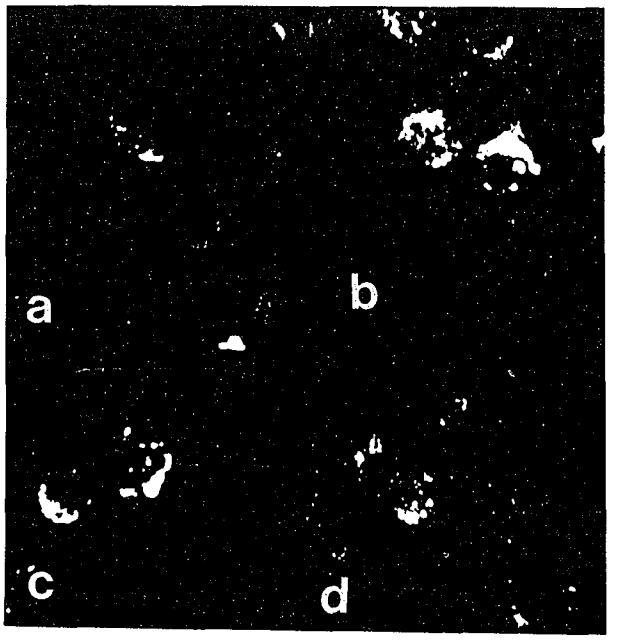


Figure 6. Uptake of heat-aggregated FITC-TT by HT29. HT29 cells grown on coverslips were incubated with either 60 μg of insoluble heat-aggregated TT or 60 μg of FITC-TT for 60 min at 37°C prior to fixation with methanol:acetic acid (3:1). The conglomerate of all sections is shown in a and c, whereas representative cross-sections of HT29 cells are shown in b and d. A decrease of uptake of heat-aggregated FITC-TT uptake was evident (a, b) when compared with the FITC-TT controls (c, d). (X100.)



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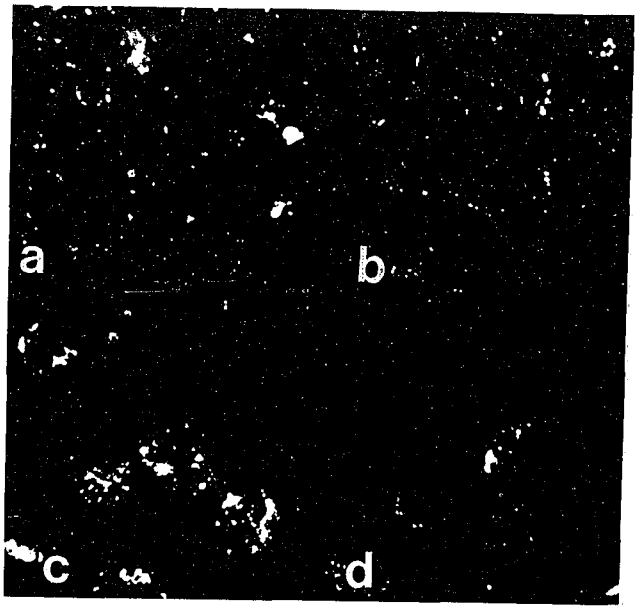

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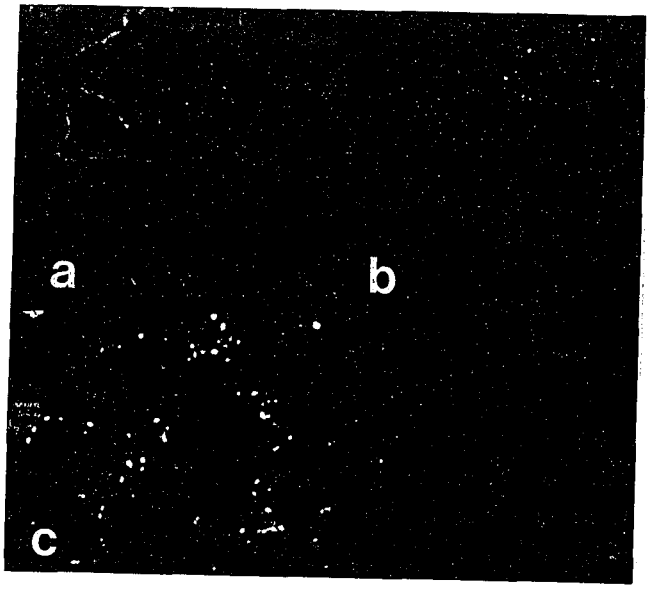

Figure 9. Kinetic studies of FITC-TT uptake by IFN-γ treated HT29. HT29 cells grown on coverslips were treated with 100 U/ml of IFN-γ for 48 h prior to incubation with 60 μg of FITC-TT for varying periods at 37°C. The cells were then washed in PBS and fixed with methanol:acetic acid (3:1). No antigen uptake was evident at 15 min (a). Intracellular signals were evident at 30 min (b). Increased fluorescence signals were evident at 60 min (c). (X100.)

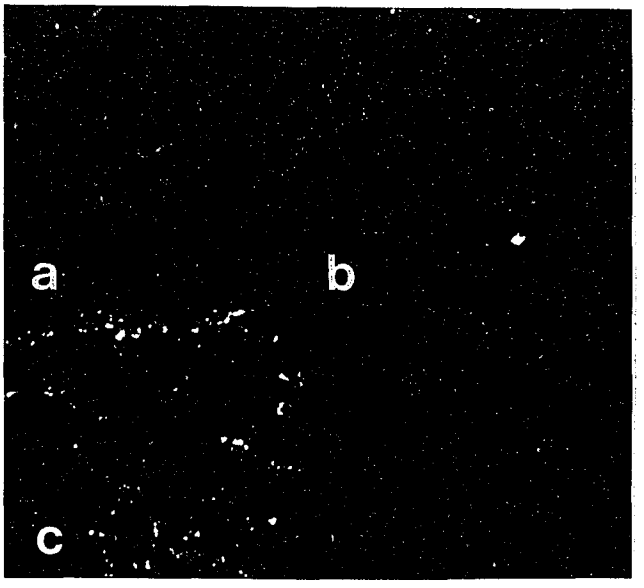

Figure 10. Kinetics studies of FITC-TT uptake by GM-CSF treated HT29. HT29 cells grown on coverslips were treated with 25 ng/ml of GM-CSF for 72 h prior to incubation with 60 µg of FITC-TT for varying periods at 37°C. The cells were then washed in PBS and fixed with methanol:acetic acid (3:1). No antigen uptake was evident at 15 min (a). Intracellular signals were evident at 30 min (b). More punctate staining was evident at 60 min (c). (X100.)

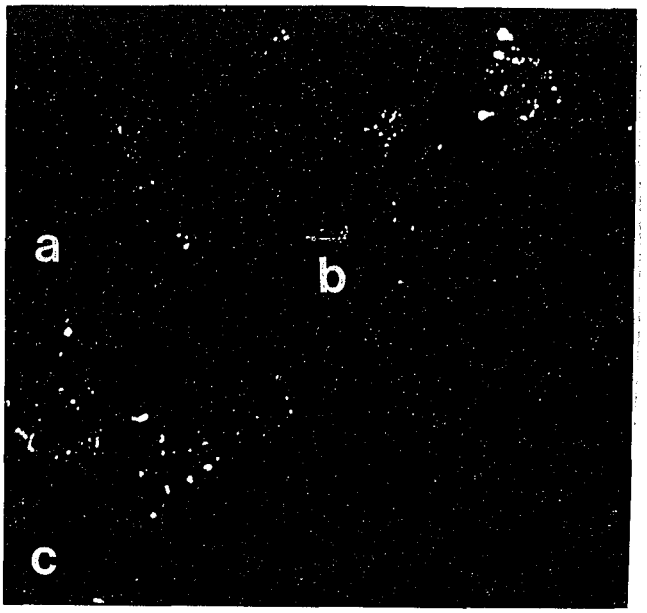

Figure 11. Uptake of FITC-TT by IFN-γ or GM-CSF treated monocytes. Monocytes incubated with IFN-γ (100 U/ml for 48 h), or GM-CSF (25 ng/ml for 48 h) were pulsed with 60 μg of FITC-TT (2 mg/ml) for 10 min at 37°C prior to fixation with methanol:acetic acid (3:1). Increased fluorescence signals were evident in IFN-γ treated (b) as well as GM-CSF treated (c) monocytes when compared with the control (a). (X100.)

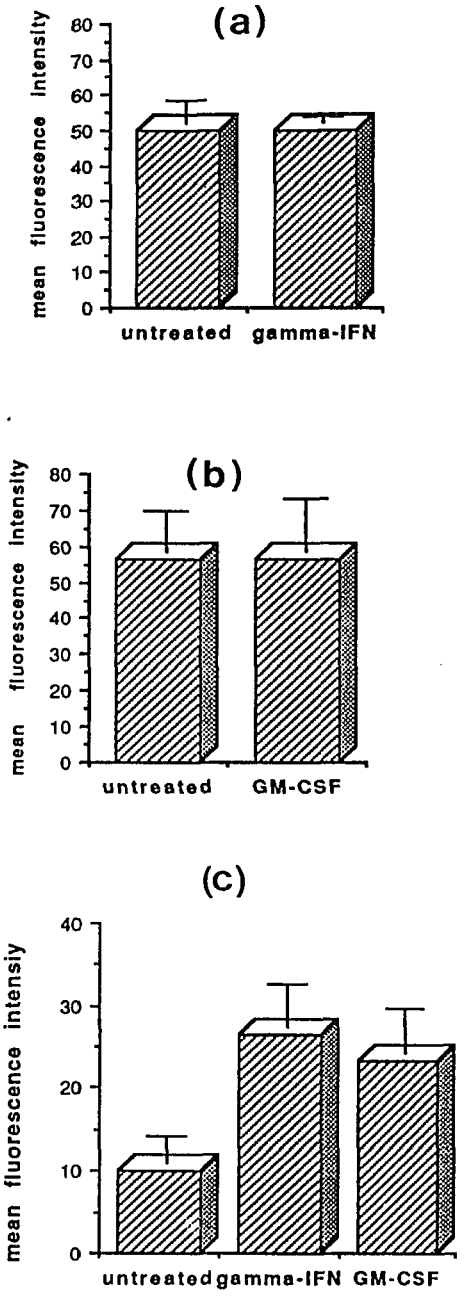

Figure 12. Measurement of FITC-TT uptake in HT29 and monocytes. Mean fluorescence intensity was measured by confocal microscopy on a per cell basis. Neither IFN-γ (a) nor GM-CSF (b) enhanced the uptake of FITC-TT, as reflected by the mean fluorescence intensity, in HT29. In contrast, there was a 2-fold increase in FITC-TT uptake in monocytes treated with these cytokines (c). One representative experiment of four.

GENERAL DISCUSSION

The GI tract is a mixed immunologic organ. Some antigens induce active immunity while others induce tolerance. One plausible scenario to help explain this dichotomy is that the way(s) in which antigen is handled by the gut will dictate the immune response. This can be looked at in one of two ways. Neither are mutually exclusive; in fact, both are interrelated. First, the type of antigen (e.g. large particulate, receptor-bound antigen or soluble antigen) might dictate the path it takes from the lumen to be presented to the cells of the mucosal immune system. Second, the cell (M cells vs. IEC) responsible for antigen sampling may dictate the response generated. It is notable in the literature that antigens which are handled by M cells usually induce active immune responses rather than immunosuppression (tolerance). This may be attributed to the fact that M cells serve as a conduit which focuses the antigen on the Peyer's patches. For antigen that is handled by the IEC, the nature of antigen appears to dictate the type of immune response mounted. In general, most protein antigens induce oral tolerance, while bacteria or viruses may elicit active immune responses. For invasive pathogens which can traverse the IEC via membrane receptors on the IEC, they can gain access to the underlying lamina propria. Therefore, they can be processed and presented by conventional APC there to stimulate CD4+ T cells, similar to pathways utilized in the systemic immune system. In contrast, most dietary antigens which consist predominantly of soluble protein antigens usually do not

elicit immune responses. Rather, they induce tolerance. Several groups including our own have documented the APC properties of IEC (Bland and Warren, 1986a; Mayer and Shlien, 1987; Kaiserlian *et al.*, 1989). Most interestingly, IEC have been shown to activate $CD8^+$ suppressor T cells. Possibly, antigen handling by IEC is different from that of conventional APC in the systemic immune system. To define the unique properties of IEC, the antigen presenting cell properties were compared and contrasted between the IEC and conventional APC in this thesis.

Our data suggest that IEC are less efficient antigen presenting cells than conventional APC with regard to antigen uptake and processing. When we examined the uptake of soluble protein antigen (e.g. TT, OVA) by the intestinal epithelial cell lines, the kinetics of uptake was slower than that of monocytes. In addition, the trafficking of antigen to lysosomal compartments in IEC was slower. These findings suggest that IEC require a longer period for presentation to occur, in contrast to macrophages which only require 20-45 min (Harding *et al.*, 1991). Furthermore, our data from subcellular fractionation assays and ^{125}I-labeled antigen processing studies show that antigen processing by the IEC is slow and limited. This finding is supported by the observation reported by Bland's group (1989) who examined the processing of OVA by rat IEC in vitro. When comparing lysates from enterocytes with lysates from splenocytes after antigen pulsing, minimal processing of OVA was observed in rat IEC. Thus, antigen processing in IEC differs from that in conventional APC. This raises the question as to whether the

peptides generated by the IEC are different from those generated by the conventional APC. Characterizing antigen processing and defining those peptides generated by the IEC might help explain why IEC stimulate CD8+ T cells while conventional APC stimulate CD4+ T cells.

Although IEC have been shown to stimulate CD8+ T cells in vitro, we show that exogenous protein antigen follows a class II pathway in the IEC. Gold-labeled or fluorescein-labeled TT was endocytosed and evident in early endosomes, then in late endosomes (multivesicular bodies), and subsequently in secondary lysosomes. Thus, similar to the findings in conventional antigen presenting cells, exogenous soluble antigens follow a classical class II pathway in the IEC. Interestingly, MHC class II molecules have been reported to be expressed on the basolateral membranes, in multivesicular bodies, and lysosomes situated in the apical cytoplasm of IEC (Mayrhofer and Spargo, 1989). Such observations raise the possibility that these compartments could be the potential sites of antigen processing and complexing of peptides with the MHC class II molecules in IEC. Although IEC have been shown to stimulate predominantly CD8+ T cells, Kaiserlian *et al.* (1989) have shown that MHC class II molecules on the IEC are functional and can present peptides derived from KLH to CD4+ T cells.

In addition, our observations from the polar transport studies support the concept that it is rare for soluble non-receptor-bound antigen to transcytose from the apical to basolateral surfaces in

adult IEC (Hughson and Hopkins, 1990). In polarized Caco-2 cells, fluorescein-labeled TT which was applied apically did not traffic to the basolateral side. This observation is confirmed by the results obtained from electron microscopic studies in Caco-2, where gold-labeled TT was also found to reside in the lysosomal compartment located in the apical cytoplasm and did not transcytose to the basolateral regions. Hence, it is possible that soluble protein antigen is processed in the late endosomal or lysosomal compartments which are located apically. Peptides derived from these compartments then complex with MHC class II molecules. Subsequently, these peptide-MHC class II complexes are transferred to another vesicular compartment and are eventually expressed on the basolateral surfaces of the cells. No direct evidence is available to support this assumption yet, but isolation of peptide-MHC complexes from these compartments might help in dissecting out the intracellular trafficking in IEC. Locating protein antigens in acidic late endosomal and lysosomal compartments may be significant with respect to the possibility of confining antigen processing and presentation of endocytosed protein antigen to the IEC. Since most soluble protein antigens are processed inside the IEC, the conventional APC in the lamina propria might have less of a chance to sample sufficient antigens to mount an active immune response. In addition, if protein antigen is processed and presented by IEC, based on previous data, $CD8^+$ suppressor T cells will get activated resulting in immunosuppression. Perhaps, this in part explains why most non-receptor-bound soluble protein antigens are weak immunogens. To explore this possibility, examination of the

intracellular trafficking of other immunogens, such as cholera toxin, which are taken up by receptor-mediated endocytosis is worthwhile. Interestingly, Lencer *et al.* (1992) failed to observed any direct transcytosis of cholera toxin from the apical to basolateral side of polarized T84 cells. Since the activation of adenylate cyclase by cholera toxin depends on a temperature-sensitive vesicular transport, they concluded that cholera toxin located in the apical endosomes might be transported to late endosomes or lysosomal compartments located apically. Thus far, although the mucosal adjuvant activity of cholera toxin is well known, the mechanisms underlying its immunogenicity are not well understood. It has been proposed that cholera toxin can enhance intestinal permeability so that more antigens can gain access to the lamina propria (Lycke *et al.*, 1991). In addition, since cholera toxin preferentially binds to the M cells, perhaps it is transported to the underlying macrophages for conventional antigen presentation to activate lymphocytes in the Peyer's patches (Elson and Dertzbaugh, 1994). From our standpoint, whether an antigen is immunogenic or tolerogenic may relate to whether it is taken up by fluid phase endocytosis or receptor-mediated endocytosis. To date, no evidence is available on how cholera toxin affects other soluble antigen uptake by the IEC or how it traffics intracellularly in the IEC. Since several groups have reported that cholera toxin can abrogate tolerance to a bystander soluble protein antigen (Liang *et al.*, 1988; Pierre *et al.*, 1992), it would be of interest to examine the antigen uptake and intracellular trafficking of a protein antigen that is either conjugated to or mixed with cholera toxin. If cholera toxin can undergo apical to

basolateral transcytosis like other receptor-bound proteins, it might be able to gain access to the lamina propria and elicit active immune responses. Examining the uptake and intracellular trafficking of cholera toxin or cholera toxin-conjugated antigen might shed light on the mechanisms responsible for the adjuvant activity of cholera toxin.

Lastly, our data show that antigen uptake by the IEC is a stable process. Fluid phase endocytosis in the IEC was affected by the physical nature of antigens rather than the size of antigens. IEC lines are capable of taking up soluble proteins of different sizes like TT, OVA, KLH, and IgG with similar kinetics. Because of its nonphagocytic nature, IEC are incapable of internalizing insoluble immune complexes or particulate antigens with the exception of some enterobacteria which express specific surface proteins that bind to the surface of epithelium and trigger phagocytosis (Pulkkinen and Miller, 1991; Pepe and Miller, 1993). Since HT29 cells do not express Fcγ receptor, uptake of soluble IgG immune complexes was comparable to other soluble proteins. Unfortunately, we did not examine the uptake of insoluble or soluble IgA immune complexes since polymeric IgA antibodies against soluble proteins are not available. Kaetzel *et al.* (1992) have reported that soluble IgA immune complexes can transcytose from the basolateral to apical side of IEC to help eliminate immune complexes formed in the lamina propria. Since IEC including HT29 cells express pIgR (Mostov and Blobel, 1982), it would be of interest to examine if insoluble IgA immune complexes applied apically or basolaterally could be taken

up by the IEC and transcytose through the IEC. The formation of insoluble IgG immune complexes in the lumen might be a blessing or a curse. Our data suggest that insoluble IgG immune complexes might help limit antigen entry through the intestinal epithelium. On the contrary, it has been shown that in certain GI disease states, nonspecific deposition of excess IgG immune complexes on the intestinal epithelium activates complement resulting in damage to the intestinal epithelium (Halstensen *et al.*, 1993). Lastly, we demonstrated that prophagocytic cytokines such as IFN-γ or GM-CSF did not affect the kinetics or quantity of antigen uptake by the IEC although we have reported that freshly isolated intestinal epithelial cells and HT29 cell line express receptors for IFN-γ and GM-CSF and these cytokines are functional in that they can augment the expression of MHC class II molecules on IEC (Panja *et al.* 1994). Again, the lack of effect of antigen uptake might be attributed to the nonphagocytic nature of IEC or the cytoskeletal elements of IEC which might be distinct from those of monocytes This non-responsiveness might be beneficial to the well-being of the intestinal epithelium because these prophagocytic cytokines are constitutively secreted by mucosal lymphocytes (IEL and LPL) and the IEC.

In this thesis, human intestinal epithelial cell lines were utilized as model systems to examine antigen uptake and trafficking. Although intestinal epithelial cell lines might differ from their non-malignant counterparts, they allow for the establishment of optimal

conditions for future studies on freshly isolated normal IEC. In conclusion, this work has resulted in the following findings:

1. IEC are less efficient antigen presenting cells than monocytes with regard to antigen uptake and processing. Whether these properties are responsible for generating specific "tolerogenic" peptides to activate $CD8^+$ suppressor T cells or down-regulating immune responses by being less efficient in antigen presentation require further investigation.

2. IEC are capable of internalizing exogenous soluble protein antigens, which follow a classical class II endocytic pathway. Since antigens internalized by fluid phase endocytosis appeared to remain in the late endosomal and lysosomal compartments, it is possible that protein antigens are processed in those sites in the IEC. Confining antigen processing to the IEC limits the access of soluble protein antigens to the conventional APC in the lamina propria, which may explain why most orally administered soluble protein antigens are poor immunogens. Previous studies have demonstrated MHC class II molecules in the late endosomal and lysosomal compartments, thus it would be of interest to examine antigen processing in these compartments and identify the antigen/peptides associated with MHC class II molecules. This might yield clues as to how IEC activate $CD8^+$ T cells.

3. Antigen uptake by IEC is a stable process and distinct from that of monocytes. Since IEC are in a unique environment where maintaining

homeostasis is the priority, antigen uptake by IEC is tightly controlled and not affected by factors that usually enhance antigen uptake in conventional APC.

Given that gut mucosal immune responses are distinct from systemic immune responses, there is an obvious dichotomy in antigen handling between the IEC and conventional APC. Characterizing antigen handling in the intestinal mucosal immune system can certainly provide better insights into the active roles that the IEC play in maintaining homeostasis and disease states.

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