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Characterization Of Intestinal Epithelial Cells In Acute Intestinal Allograft Rejection

Xian-chang Li

CHARACTERIZATION OF INTESTINAL EPITHELIAL CELLS IN ACUTE INTESTINAL ALLOGRAFT REJECTION

By

Xian-Chang Li

Department of Pathology

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Faculty of Graduate Studies
The University of Western Ontario
London, Ontario, Canada

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ABSTRACT

Intestinal transplantation (IT) remains highly experimental compared to other solid organ transplants because of poorly defined mechanisms of graft rejection. In this project, the role of luminal bacteria-derived endotoxin and inflammatory cytokines (IL-1, IL-6, TNF-α) in intestinal epithelial damage during acute graft rejection was examined. In a novel mouse model of IT, the acute intestinal rejection was associated with elevated levels of endotoxin, IL-6, and TNF-α in the peripheral blood. The progressive increase in endotoxin and TNF-α levels correlated well with the histologic severity of epithelial damage. To further define the role of endotoxin and inflammatory cytokines on intestinal epithelial cells, several epithelial cell lines from the small intestine of BALB/c mice were established by transfection of primary cultured epithelial cells with pMK16 plasmid containing the origin-deficient simian virus 40 (SV40) DNA. One epithelial cell clone (IEC-4.1) stably integrated large T gene of the SV40 virus into the cell genome. IEC-4.1 cells exhibited phenotypic and ultra-structural features of enterocytes. IEC-4.1 cells expressed well-developed microvilli on the cell surface, they formed confluent monolayers and expressed junctional protein zonula occluden-1 (ZO-1), major histocompatibility complex (MHC) class I, and low levels of MHC class II molecules. *In vitro* studies using IEC-4.1 cells demonstrated that TNF-α was highly cytotoxic to IEC-4.1

cells. Endotoxin did not affect the growth and viability of IEC-4.1 cells but it potentiated TNF-α-mediated enterocytotoxicity. We further examined the capacity of IEC-4.1 cells to generate molecules that are capable of promoting immune-mediated injury. In northern blot analysis, IEC-4.1 cells constitutively expressed IL-1α mRNA. These cells also expressed gene transcripts for IL-6, TGF-β, and adhesion molecules ICAM-1 and VCAM-1 in response to endotoxin or TNF-α stimulation. Further analysis showed that ICAM-1 and VCAM-1 had different kinetics of cell surface expression following stimulation. The expression of adhesion molecules by IEC-4.1 cells effectively promoted macrophage adhesion to the apical domain of IEC-4.1 cells and this process was also enhanced by endotoxin or TNF-α.

These data suggest that luminal bacteria-derived endotoxin may play an important role in the pathogenesis of intestinal epithelial damage. Intestinal epithelial cells may be actively involved in regulating leukocyte trafficking and leukocyte-epithelial interactions through the expression of inflammatory cytokines and adhesion molecules as well as being a vulnerable target during this process. Strategies to specifically block interactions between epithelial cells and graft-infiltrating cells may help to reduce the risk of graft damage.

DEDICATION

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LIST OF ABBREVIATIONS

APC	antigen-presenting cells
CD	cluster of differentiation antigens
DC	dendritic cells
DEPC	diethypyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DTH	delayed-type hypersensitivity
EDTA	ethylenediaminetetracetic acid
ELISA	enzyme-linked immune absorbent assay
FACS	fluorescence-activated cell sorter
FAE	follicle-associated epithelium
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GVHD	graft-versus-host disease
HBSS	Hanks' balanced salt solution
HLA	human leukocyte antigen
ICAM	intercellular adhesion molecule
IEL	Intraepithelial lymphocytes
IFN-γ	interferon-gamma
IgA	immunoglobulin A
IL	interleukin
LPS	lipopolysaccharide
IMEM	Iscove's modified Eagle's medium
IT	intestinal transplantation
LAK	lymphokine-activated killer cells

LFA	leukocyte function-associated antigen
mRNA	messenger ribonucleic acid
MLN	mesenteric lymph nodes
MHC	major histocompatibility complex
ml	milliliter
mM	millimolar
ng	nanogram
OD	optical density
PP	Peyer's patches
POD	post operative day
PGE_2	prostaglandin E_2
SD	standard deviation
SDS	sodium dodecyl sulfate
SV40	simian virus 40
TCR	T cell receptor
TGF	transforming growth factor
TNF	tumor necrosis factor
TPN	total parenteral nutrition
U	unit
uCi	microCurie
ul	microliter
ug	microgram
VCAM	vascular adhesion molecule
VLA	very late antigen
ZO-1	zonula occluden-1

CHAPTER 1. LITERATURE REVIEW

1.1. BRIEF OVERVIEW OF ORGAN TRANSPLANTATION

The first attempts at transplantation date back to ancient times. The earliest record of autogenous pedicled grafts from the forehead, neck, and cheek to restore mutilations of the nose, ear, and lip can be found in a Sanskrit text from India (Flye, 1989). In the mid 1700s, it was recorded that John Hunter successfully replanted the first premolar of a patient within hours of having it knocked out (Kahan, 1990). Solid organ transplantation with blood vessel anastomoses, however, was not attempted until the early 1900s. The real impetus behind modern transplantation was the Second World War and the Battle of Britain. The Royal Air Force pilots were often severely burned when their planes crashed. It was noticed at that time that the burned area of the body was directly correlated with the mortality of the pilots. For this reason, British doctors turned to skin transplantation from other human donors as a mode of therapy. However, attempts to replace damaged skin with skin from unrelated donors were uniformly unsuccessful. The skin grafts always died and peeled off within a short period of time. This problem led many investigators including Sir Peter Medawar to study skin transplantation in animal models. The concept of an immunologic basis of transplantation introduced by Medawar

and colleagues (1953) greatly advanced the theory and practice of organ transplantation. The introduction of effective immunosuppressive agents such as anti-metabolites, steroids, and more recently, cyclosporins, anti-lymphocyte globulin, and FK506, has allowed a far more practical approach to organ transplantation. Today, transplantation of kidney, liver, heart, heart-lung, and bone marrow has become reliable therapeutic procedures for end-stage organ failure with well over 80% survival in the first year of transplantation (Alexander, 1990). Pancreas transplantation has advanced from an experimental procedure to a therapeutic alternative (Sato et al., 1989). Intestinal transplantation (IT), however, still remains highly experimental due to difficulties in controlling rejection (Todo et al., 1992b). A better understanding of the immunologic basis of intestinal graft rejection and the development of more effective and specific immunosuppression remain elusive goals.

1.2. INTRODUCTION OF INTESTINAL TRANSPLANTATION

Interest in IT has been fueled by attempts to find an alternative therapeutic modality to the problem of short bowel syndrome. The short bowel syndrome refers to a collection of symptoms caused by a significant loss of the small intestine. This situation leads to an urgent clinical condition manifested by dehydration, severe weight loss, fatigue, lassitude, weakness, and even death

(Vanderhoof et al., 1992). The severity of symptoms is related to the extent and the specific levels of the small bowel removed. The loss of small amounts of small intestine usually causes no clinical symptoms, since the absorptive and digestive surface of the small intestinal mucosa is more than adequate to maintain nutrition. Prolonged survival with oral alimentation has been reported in a number of patients who have the intact duodenum and as little as 15 to 45 centimeters of residual jejunum (Purdum et al., 1991). However, without long-term total parenteral nutrition (TPN) less than 60 centimeters of jejunum or ileum, in addition to the entire duodenum, causes the reduction of absorption of virtually all nutrients including water, electrolytes, fat, protein, carbohydrate, vitamins, and trace elements (Ishii et al., 1993).

The most common cause of short bowel syndrome is vascular accident such as thrombosis of the superior mesenteric blood vessels, low flow bowel ischemia, volvulus, and strangulated intestinal obstruction. Less common causes include trauma, Crohn's disease, neoplasm, necrotizing enterocolitis, and congenital anomalies. Patients with short bowel syndrome are usually maintained on home TPN. There are many limitations about this therapy although patients can survive for years (Jejeebhoy et al., 1976; Steiger et al., 1983; Bowyer et al., 1985). Many patients suffer from debilitating diarrhea. Opportunities to work and travel are severely restricted by the requirement to infuse a

intravenous solution every 8 to 12 hours daily. The intravenous catheters have to be removed frequently because of infection or thrombosis. Patients, particularly children, often develop TPN-induced liver disease. Some patients die from starvation when all sites for intravenous access have been exhausted. Treatment with home TPN costs $50,000 tO $100,000 per year per patient (Wateska et al., 1980). The lifestyle restrictions, the high rate of complications, and the expense associated with home TPN have stimulated the active search for an alternative form of therapy.

It was realized more than three decades ago that IT would be the ideal solution for end stage intestinal failure. Lillehei and colleagues (1959) first described a surgical technique for small bowel transplantation in dogs. The procedure involved the transplantation of the entire small intestine with vascular anastomoses between the superior mesenteric vessels of the grafts and the respective vessels of the recipients. Recipients of isografts survived indefinitely, whereas allografts were rejected within 10 days (Octavio-Ruiz et al., 1972). Transplantation of an intestinal segment as a Thiry-Vella loop in the abdomen of dogs with anastomoses of the intestinal vessels to the iliac vessels was described by several investigators (Ruiz et al., 1972; Dennison et al., 1987; Diliz-Perez et al., 1984; Craddock et al., 1983). Similar models have been reported in pigs (Stauffer et al., 1978). Given the complexity of large animals, studies using large animal models were limited to

the histological and functional characterizations of the intestinal grafts. With the introduction of a rat model of IT in 1971, studies on the underlying immunologic mechanisms of intestinal rejection began.

Monchick and Russell (1971) first described a heterotopic model of IT in rats, which involved the transplantation of the entire small intestine as a Thiry-Vella loop with the native intestine left intact. Kort (1973) described a surgical model for orthotopic IT with vascular anastomoses between the mesenteric artery and abdominal aorta, and between the portal vein of the graft and that of the recipient. The native sma.. intestine was removed and the intestinal continuity was restored with the graft.

In the orthotopic model, recipient survival is dependent entirely upon the function of the intestinal graft, which is closer to the clinical and physiologic situation. The heterotopic model gives the advantage of accessibility to the grafts for histologic and immunologic evaluations. Although the orthotopic model may be the preferred one, the reported longer operating time and higher incidence of complications have resulted in a number of studies in which the heterotopic model is used (Grant et al., 1991b).

Rejection of intestinal allografts presents some unique characteristics. In contrast to renal or cardiac allografts where depletion of passenger leukocytes significantly prolongs graft survival, and in some animal strain

combinations, induces long-term acceptance of the allografts (McKenzie et al., 1984b), the passenger leukocytes in the intestinal grafts appear to be non-essential in inducing graft rejection, since depletion of lymphoid cells from intestinal grafts fails to prevent intestinal allograft rejection (Stangl et al., 1990; Gundlach et al., 1991). This may be due to the difficulty in depleting all of the lymphoid cells in large grafts such as small intestine given the residence of these cells in extravascular locations. It is also likely that other elements within the small intestine, in addition to passenger leukocytes, may play a critical role in intestinal allograft rejection.

Intestinal rejection is associated with intense, bi-directional cell trafficking. This process is characterized by the progressive emigration of donor lymphoid cells from the intestinal graft into the recipient lymphoid tissues, while the recipient mononuclear cells repopulate the intestinal graft (Arnaud-Battandier et al., 1986; Iwaki et al., 1991). In humans, infiltration of CD25+ (IL-2 receptor) T cells into the lamina propria and the *de novo* expression of major histocompatibility complex (MHC) class II antigens by crypt cells are probably the earliest histologic signs of intestinal rejection (Cerf-Bensussan et al., 1990). In rats, the initial rejection process takes place in the graft mesenteric lymph nodes (MLN) and Peyer's patches (PP), followed by pericryptal cellular infiltration in the lamina propria. Immunohistochemistry and phenotypic

studies demonstrate that macrophages dominate the cellular infiltrate throughout the rejection process (Ingham-Clark et al., 1990; Clark et al., 1990; Grover et al., 1993). The increase in the macrophage population is consistently accompanied by a concurrent increase in the T helper cell subset (W3/25), suggesting the possible role of a delayed-type hypersensitivity (DTH) response in the pathogenesis of intestinal allograft rejection (Kim et al., 1990). The appearance of cytotoxic T cells (OX-8) is a late event when clinical rejection is evident (Kim et al., 1990).

1.3. GRAFT-VERSUS-HOST DISEASE AND INTESTINAL TRANSPLANTATION

Graft-versus-host disease (GVHD) is initiated by graft-derived immunocompetent cells that recognize and destroy organs of recipients. The basic conditions essential for the development of GVHD include: (1) histoincompatibility between a donor and a recipient; (2) presence of immunocompetent cells in a graft or inoculum; and (3) an immunologically compromised recipient (Ferrara et al., 1991).

GVHD can be classified into acute and chronic categories on the basis of histologic patterns and clinical presentations. Acute GVHD involves epithelial necrosis in the three principal target organs: skin, liver, and gastrointestinal tract (Slavin et al., 1973). In the liver, the biliary epithelial cells, but not the hepatocytes, are

involved. Clinically, acute GVHD is characterized by skin rash, jaundice, and diarrhea. When the epithelial necrosis is extensive, the skin or the gut epithelium may simply slough. In this condition, acute GVHD is fatal. Chronic GVHD is characterized by fibrosis and atrophy of one or more of the same organs without evidence of cell necrosis (Gleichmann, 1986). Occasionally, fibrosis and necrosis can be present at the same time, leading to a diagnosis of acute and chronic GVHD. Chronic GVHD may lead to complete dysfunction of the affected organ and may also be fatal.

Both acute and chronic GVHD are commonly treated with high doses of steroids, with or without other immunosuppressive drugs, but neither condition responds well to this treatment (Barrett, 1987). Much effort has been focused on the prevention of GVHD. In bone marrow transplantation, the risk of development of GVHD is related to the antigenic disparity between the donor and recipient, the number of immunocompetent cells transferred, the presence of bacterial infection, and the age of the recipient (Barrett, 1987). HLA typing is the most effective means of preventing GVHD in humans.

In animal models, acute GVHD is initiated by mature T cells derived from the graft. In bone marrow transplantation, elimination of mature T cells from the inoculum can effectively prevent the development of GVHD (Korngold et al., 1987). In humans, efforts to eliminate T cells from bone marrow have reduced the incidence of GVHD,

but also reduced the efficacy of stem cell engraftment. The mature T cells, perhaps through the production of cytokines such as IL-3 and granulocyte-monocyte colony-stimulating factor, improve the process of stem cell repopulation (Ferrara et al., 1985).

Although GVHD is initiated by alloantigens expressed on host cells, the effector mechanisms that mediate epithelial cell necrosis are less well defined. In bone marrow grafts, mature T cells can mediate epithelial damage by releasing lymphokines, particularly tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), upon activation (Mizuno et al., 1986; Piguet et al., 1987). In some animal models, cytotoxic T cells play an important role in tissue damage (Mowat et al., 1982). Natural killer (NK) cells have been shown to be the effector cells in mediating cell necrosis during GVHD (Ghayur et al., 1987a; Ghayur et al., 1987b). It has been proposed that NK cells, activated by the locally produced IL-2, differentiate into lymphokine-activated killer (LAK) cells that attack epithelial cells. In contrast to cytotoxic T cells, NK cells and LAK cells can lyse normal cell types and are not restricted by MHC molecules.

The intestine is populated by a large number of immunocompetent cells; it is not surprising that IT is capable of inducing GVHD (Kirkman et al., 1984). It appears that intestinal rejection and GVHD exist in a dynamic equilibrium, since measures that reduce the risk of intestinal rejection seem to increase the incidence of GVHD.

In animal models, GVHD is easily demonstrated in F1-hybrid rats transplanted with the parental strain intestinal grafts (Grant et al., 1989b). In the absence of immunosuppression, overt GVHD will develop within 15 days after IT. Clinically, the animals develop hunched posture, erythema of the paws and ears, and significant weight loss. Most animals die of systemic sepsis following bacterial translocation from the gut (Deitch et al., 1987). GVHD induced by IT appears to be particularly intense within the host spleen, since spleen enlargement is very prominent, and is sometimes used as a criterion to determine the severity of GVHD (Hard, Jr. et al., 1987).

The mature T cells in mucosal lymphoid tissue, particularly those in the MLN, are essential for the development of GVHD after IT (Wallander et al., 1989). In certain rat strain combinations, GVHD can be prevented by surgical removal of the graft MLN prior to transplantation (Luck et al., 1990; Saat et al., 1990). Selective depletion of T lymphocytes from intestinal grafts also prevents the development of GVHD (Shaffer et al., 1991; Smith et al., 1992; Clark et al., 1992). The size and origin of intestinal grafts transplanted do not affect the incidence of GVHD, but do affect its severity. In general, a shorter intestinal graft is associated with better host survival following the development of GVHD after IT (Kimura et al., 1986).

While GVHD has attracted considerable research interest, it has not been a major barrier to IT in large animals

including humans. First, severe GVHD rarely occurs in higher order, outbred species after IT (Schraut, 1988). Second, mild GVHD can compromise the recipient's immune system, and therefore, may actually help to delay or prevent intestinal allograft rejection (Grant et al., 1990; Saat et al., 1990). Third, GVHD can be effectively ameliorated by several techniques such as donor pre-treatment with anti-lymphocytic products (Diflo et al., 1988).

1.4. CLINICAL INTESTINAL TRANSPLANTATION

In the late 1960s and early 1970s, seven patients who were treated with azathioprine, steroids, and anti-lymphocyte globulin had intestinal transplants, but none survived. It was clear that vigorous graft rejection and severe sepsis encountered after intestinal grafting were beyond the control of the agents offered at that time. The initial enthusiasm for small bowel transplantation waned. After the introduction of cyclosporin A in the late 1970s, prolonged intestinal allograft survival was achieved in several animal models (Aeder et al., 1984; Craddock et al., 1983), and in some species, cyclosporin A induced a long-term acceptance of intestinal grafts (Kirkman et al., 1984; Madara et al., 1985). With the encouragement of experimental IT, at least 13 patients had undergone isolated IT up to 1991 with cyclosporin A as the primary immunosuppressive drug. However, the results were disappointing: two had partially functioning grafts; eight

had their grafts removed because of rejection or technical complications; and two patients died from sepsis or unexplained causes (Cohen et al., 1986; Grant, 1989a; Watson et al., 1989). None of these patients was able to stop parenteral supplementation and resume a normal diet.

The introduction of the more potent immunosuppressive agent FK506 was a major advance in IT (Casavilla et al., 1992; Todo et al., 1992a; Tzakis et al., 1992). A total of 59 intestinal transplants (32 children and 27 adults) have been performed by November, 1993 at the University of Pittsburgh (Todo et al., 1994). The immunosuppressive protocol developed by the University of Pittsburgh involved the use of a FK506-prednisone-prostaglandin cocktail and an anti-CD3 monoclonal antibody (OKT3) (Todo et al., 1992b). Despite this powerful immunosuppression, intestinal rejection has continued to be a major barrier of clinical IT. Almost all the intestinal recipients had one or more episodes of rejection and the rate of graft loss due to acute rejection has been over 27%. The 2 year patient survival rate is 80% and the graft survival rate is only 53%. The use of FK506 has created significant renal, neural, and endocrine toxicity. All the intestinal graft recipients experienced one or more episodes of bacterial, viral, or fungal infection (McAlister et al., 1993b). The incidence of lymphoproliferative disorders associated with IT has been over 15%. The current experience at the University of Pittsburgh is that about 50% of the intestinal recipients

resumed an oral diet, at least for a brief period of time; 30% died; and 20% of the patients returned to TPN after graft failure (Todo et al., 1994). Some of the patients with long-term surviving intestinal grafts have had poor graft function (Todo et al., 1992b).

1.5. IMMUNOLOGICAL BASIS OF GRAFT REJECTION

1.5.1 Major histocompatibility complex

Major histocompatibility antigens are cell surface glycoproteins encoded by a set of closely linked genes called MHC. The MHC is mapped to the short arm of chromosome 6 in humans and chromosome 17 in mice. The MHC is referred to as the human leukocyte antigen (HLA) complex in humans, H-2 complex in mice, and RT1 in rats.

MHC antigens are highly polymorphic molecules that can be grouped into class I or class II on the basis of structure, function, and tissue distribution. MHC class I molecules are formed by a polymorphic heavy chain encoded by the MHC class I genes and a relatively invariant polypeptide termed β_2-microglobulin encoded on a separate chromosome. The heavy chain of MHC class I molecules is about 45,000 daltons and encoded by genes in the MHC region. The dominant MHC class I genes are HLA-A, HLA-B, and HLA-C in humans, and H-2 K, H-2 D in mice. In addition, three other loci, HLA-E, F, and G, have been identified in humans in the class I region, but the antigens they encode are far less polymorphic than HLA-A, HLA-B, or HLA-C.

MHC class II genes are located in the HLA-D region of MHC in humans and H-2 I region in mice, in a chromosomal length about 1,100 kilobase pairs, at the centromeric end of the MHC. The HLA-D region is divided into three major subregions: HLA-DR, HLA-DQ, and HLA-DP. In mice, the H-2 I region is divided into I-A and I-E subregions. Each subregion contains multiple genes. The MHC class II molecule expressed on the cell surface consists of an α chain and a β chain encoded by genes in the MHC class II region (Touraine et al., 1989). Each MHC class II expressing cell can potentially express products from each of these subregions. In most of these cells, DR molecules are expressed at higher levels than DQ or DP molecules (Touraine et al., 1989). The rat RT1 antigens have been demonstrated to have similar characteristics to H-2. However, at the gene level, the genetic polymorphism of RT1 is significantly lower than H-2 or HLA.

MHC class I and MHC class II molecules have different patterns of tissue distribution. The MHC class I molecules are constitutively expressed by most nucleated cells. In contrast, the MHC class II molecules are expressed only by a small number of cell types such as B cells, macrophages, monocytes, and dendritic cells (DC) (Touraine et al., 1989).

Both MHC class I and class II molecules bind peptide antigens to form a MHC-peptide complex that can be recognized by the T cell receptors (TCR). T cells that express CD8 on their surfaces recognize peptides bound to

MHC class I molecules. CD8+ T cells also respond directly to allogeneic MHC class I molecules. As part of this interaction, the CD8 molecule binds to a non-polymorphic region of MHC class I molecule on target cells. Thus CD8+ T cells are MHC class I-restricted. CD4+ T cells recognize antigens bound to MHC class II molecules and respond directly to allogeneic MHC class II molecules. The CD4 molecule binds to a non-polymorphic region of the MHC class II molecule expressed by the antigen-presenting cells (APC) (Krensky et al., 1990).

1.5.2. Mechanisms of allograft rejection

1.5.2.1. The induction phase of graft rejection

According to the current dogma, rejection is initiated through interactions between APC and T lymphocytes. Passenger leukocytes, particularly DC, are one of the major types of APC in graft rejection (Lechler et al., 1983; Lechler et al., 1982). It has been shown that removal of passenger leukocytes from islet (Faustman et al., 1981), thyroid, heart (McKenzie et al., 1984a), liver, and kidney grafts (McKenzie et al., 1984b) prolongs allograft survival in rats. Replacement of donor passenger leukocytes with recipient phenotype by bone marrow reconstitution has prolonged kidney graft survival in rats (Guttmann et al., 1969) and dogs (Rapaport et al., 1988). Attempts to remove passenger leukocytes from human renal grafts yield similar results (Guttmann et al., 1984). Recent studies demonstrate

that graft-derived peptides can be processed and presented to CD4+ T cells by recipient APC in the context of self MHC class II molecules leading to specific T cell activation and graft rejection (Golding et al., 1984). Furthermore, parenchymal cells of an allograft have been shown to contribute to the immunogenicity of a graft provided the parenchymal cells express, or can be induced to express, MHC class II molecules (Goebels et al., 1992; Miltenburg et al., 1989).

At the molecular level, interactions between APC and T cells involve the MHC molecules and the TCR. The TCR is clonally distributed and consists of an α chain and a β chain that form a disulfide-linked heterodimeric structure on T cells. Each TCR contains a proximal constant and a distal variable region that is analogous to the B cell surface immunoglobulin. The TCR is noncovalently associated with CD3 molecules to form a CD3-TCR complex on the cell surface. CD3 is a complex of 5 polypeptides: γ, δ, ε coupled either to a homodimer of $\xi\xi$ or a heterodimer of $\xi\eta$. Once the TCR binds an antigen, the CD3 complex is thought to transmit a signal to the cell interior that contributes to T cell activation (Kuby, 1992). The CD3-TCR complex allows specific recognition of foreign peptides in association with MHC molecules. In allograft rejection, however, allogeneic MHC molecules are thought to serve as both the presenting molecules and as foreign peptides; thus, allogeneic MHC

molecules are recognized by, and stimulate, a much higher percentage of T cell clones (Chandler et al., 1993).

In addition to the CD3-TCR complex, several other accessory molecules are important in MHC and TCR interactions. Some of these molecules on T cells bind ligands on APC or target cells and provide stabilizing adhesive forces. For example, CD4 and CD8 molecules expressed on subsets of mature T cells bind non-polymorphic determinants of MHC class II on APC and MHC class I on target cells respectively. Both CD4 and CD8 function to increase the avidity of the interaction between the TCR and the antigen-MHC complex. In the cell membrane, CD4 and CD8 are associated with the TCR and can comigrate with the TCR-CD3 complex. The cytoplasmic domains of CD4 and CD8 are both associated with $p56^{lck}$, a *src*-related protein kinase that can phosphorylate the γ, δ, ε, and ξ chains of CD3, suggesting that CD4 and CD8 may play an additional role in signal transduction (Kuby, 1992). Other accessory molecules with known adhesive properties include CD2, which binds to lymphocyte function-associated antigen-3 (LFA-3); and LFA-1, which binds to intercellular adhesion molecule-1 (ICAM-1) or ICAM-2. Accessory molecules expressed on the surface of T cells may also transduce an activating or regulatory signal. CD28, which binds to B7/BB1 on the APC, can deliver a costimulatory signal in T cell activation (Harding et al., 1992; Damle et al., 1992; Koulova et al., 1991).

The binding of MHC-antigen complex by the TCR, in the presence of a second costimulatory signal, results in a cascade of molecular and cellular responses leading to T cell activation (Snell, 1957). The earliest events in this cascade are membrane phospholipid hydrolysis, elevated protein kinase C activity, and a rise in cytoplasmic calcium. During this process several proteins are phosphorylated, which in turn stimulate the transcription of genes leading to T cell proliferation and clonal expansion.

An early event in T cell activation is the production of cytokines (Krensky et al., 1990). IL-2, which is the first lymphokine released by activated CD4+ T cells, plays a critical role in the proliferation and maturation of a number of cell types. IL-2 augments lymphokine production including its own by activated CD4+ T cells. IL-2 promotes the proliferation and differentiation of cytotoxic T cells and NK cells, and also promotes B cell differentiation and antibody production. Among other cytokines produced by activated T cells, IFN-γ plays an important role by increasing the expression of MHC class I and MHC class II antigens on a variety of cell types. The increased expression of MHC antigens amplifies both the recognition and destruction of an allograft. IFN-γ also augments the production of TNF-α; induces macrophage activation; and enhances the activation of cytotoxic T cells and NK cells. TNF-α, mainly produced by macrophages and monocytes, has numerous effects that make this cytokine an important

molecule in graft destruction. Systemically, TNF-α is a potent pyrogen and promotes an acute phase reaction (Perimutter et al., 1986). The local tissue damage caused by TNF-α is primarily mediated by the activation of inflammatory cells, endothelial cells, and certain lymphoid cells. At high concentrations, TNF-α is cytotoxic to endothelial cells. TNF-α augments cytotoxic T cell and NK cell activity (Wright et al., 1987); upregulates MHC antigen expression; enhances prostaglandin production; and stimulates procoagulant activity. Furthermore, TNF-α also upregulates adhesion molecule expression on endothelial cells; stimulates leukocyte adherence; induces IL-1 and IL-2 production, and in turn, IL-2 stimulates TNF-α production from monocytes. TNF-α also induces endothelial cells to release platelet-derived growth factor, which may be involved in ischemic damage of the graft.

1.5.2.2. The effector phase of graft destruction

T cells play a central role in allograft rejection, as demonstrated by the failure of neonatally thymectomized or congenitally athymic nude rodents to mount a graft rejection response (Hall et al., 1978). Peripheral T cells fall into two subsets based on the phenotype and function. CD4+ T cells generally function as helper cells and are MHC class II-restricted. T cells that express CD8 molecules mediate suppression and cytotoxic activity, and are MHC class I-restricted.

Direct cytotoxicity mediated by activated CD8+ cytotoxic T cells is critical in allograft destruction (Mason et al., 1984). This process requires direct contact between the cytotoxic T cells and the target cells. The killing of target cells is mediated by the release of preformed membrane-disrupting proteins such as perforin. Perforin is structurally similar to the C5b-C9 "membrane attack complex" of the complement cascade (Shinkai et al., 1988). The functional maturation of CD8+ T cells is dependent upon the activation of CD4+ T cells in majority of rodent strains and in humans. Upon activation, CD4+ T cells produce cytokines such as IL-2, IFN-γ, and TNF-α that are essential for clonal expansion and functional maturation of CD8+ T cells (Bumgardner et al., 1989). In some rodent strains, however, CD8+ T cells are functionally heterogenous and some of them are able to produce IL-2 whereby sustaining the proliferation and the subsequent maturation of cytotoxic T cells (Bottomly, 1988).

The relative importance of CD4+ versus CD8+ T cells in allograft rejection is determined by: (1) the MHC class I versus MHC class II disparity between the donor and recipient; (2) the dependence of CD8+ T cells on CD4+ T cells in functional maturation; and (3) the activation status of CD8+ T cells. Synergy between CD4+ and CD8+ subsets has been demonstrated for the rejection of both MHC class I and class II mismatched allografts. The rejection of skin grafts with non-MHC incompatibility also requires the

participation of both CD4+ and CD8+ T cells (Hall, 1991). In irradiated DA rats, it has been shown that both CD4+ and CD8+ cells restore graft rejection more effectively than CD4+ cells alone (Hall et al., 1985). CD8+ T cells are effective in rejection of MHC class I incompatible grafts (Lowry et al., 1985). CD8+ T cells that have been previously sensitized can effectively mediate kidney, heart, thyroid, or pancreas graft rejection (Stepkowski et al., 1986). The dependence of CD8+ T cells upon CD4+ T cells for activation has clinical significance. In particular, matching for MHC class II or suppression of CD4+ cells may have a great impact if a rejection response is dependent on CD4+ T cell activation. Experimental studies suggest that MHC class II matching may protect class I incompatible grafts from rejection (Hall, 1991).

The inflammatory response, involving tissue injury mediated by soluble mediators, recruitment and activation of non-specific inflammatory cells such as macrophages and polymorphonuclear cells, represents a second major mechanism of graft destruction (Mason et al., 1984). Macrophages play a key role in this process. Once activated by T cell-derived cytokines, macrophages can produce inflammatory cytokines (IL-1, IL-6, and TNF-α), eicosanoids, and reactive oxygen free radicals. IL-1 and TNF-α activate blood vessel endothelial cells and promote leukocyte adhesion and migration to the graft. These cytokines also activate platelets and coagulation system leading to graft ischemia

and endothelial damage. The activation of NK cells by T cell-derived cytokines and the local production of cytotoxic cytokines such as TNF-α and TNF-β also contribute to the graft damage.

The role of antibody in acute allograft rejection is probably limited to the hyperacute rejection characterized by rapid thrombotic occlusion of the graft vasculature, which begins within minutes after restoration of blood supply to the graft. The hyperacute rejection is mediated by pre-existing donor specific antibodies. The pre-formed donor specific antibodies bind to MHC antigens expressed by the vascular endothelium of the graft, resulting in activation of the complement system. The activation of the complement system results in virtually immediate platelet aggregation and endothelial destruction in the microcirculation and subsequent loss of endothelial integrity. Clinically, the hyperacute rejection can be avoided by screening for cytotoxic antibody to donor lymphocytes prior to transplantation. Recently, it has been suggested that antibody production may play an important role in the initiation of chronic rejection.

1.6. GUT-ASSOCIATED LYMPHOID TISSUE

The lymphoid tissue that forms the intestinal immune system consists of PP, isolated lymphoid follicles, the appendix, MLN, and lymphoid cells that are scattered throughout the wall of the intestine, both in the lamina

propria and in the epithelium. It has been proposed recently that lymphoid cells within the intestinal lumen may form an another population of functional immunocytes (Reinhardt, 1984).

1.6.1. Peyer's patches

PP are collections of lymphoid nodules present throughout the small intestine from the pylorus to the ileocecal valve, but most prominent in the ileum. PP extend through the mucosa into the submucosa of the small intestine (Rothkotter et al., 1990). Human PP are well developed early in fetal life, but germinal centers in the lymphoid follicles continue to develop after birth, and the number and size of PP continue to increase until 12 to 14 years of age (Brandtzaeg et al., 1989). The development of PP after birth requires microfloral colonization of the intestinal lumen, since PP in germ-free animals are quite small, but they enlarge rapidly when animals are brought to conventional conditions (Woolverton et al., 1992).

B cells comprise about 60 to 70% of the cells in PP and are primarily located in the germinal centers. The majority of B cells bear surface immunoglobulin A (IgA) (Mayer, 1988). B cells do not differentiate into IgA-secreting plasma cells within PP. Instead, B cells primed in PP exit and migrate through lymphatics and the peripheral circulation before homing to the lamina propria where they differentiate into mature antibody-secreting plasma cells (Brandzaeg et al., 1990; Campbell et al., 1974). T cells

populate the perifollicular areas and constitute the remaining 25 to 35% of cells in PP. A few T cells are found in the lymphoid follicles and germinal centers. T cells and cytokines derived from T cells play an important role in isotype-switching from IgM to IgA-producing B cells in PP (Beagley et al., 1987; Campbell et al., 1974).

Accessory cells, defined as cells capable of presenting antigens to T lymphocytes, have been found in PP. Histologically, DC and macrophages are present in the dome region of PP, and MHC class II antigen positive cells displaying DC morphology are also found in the T and B cell zones of PP (Soesatyc t al., 1990; McKenzie et al., 1984a). DC isolated from PP are capable of presenting antigens *in vitro* (Spalding et al., 1983; Li et al., 1992).

PP function as antigen sampling sites, and therefore, play an important role in the initiation of mucosal immune responses. This is supported by the finding that certain antigens introduced into isolated intestinal loops elicit specific secretory antibody responses only when PP are present in the loop (Cebra et al., 1977). Antigens enter PP through the specialized follicle-associated epithelium (FAE). FAE differs from the surrounding epithelium. It contains "M" cells between columnar enterocytes and lacks mucus-producing goblet cells. The "M" cells originate from adjacent crypt epithelium and lack fully developed microvilli on their luminal surface (Reinhardt, 1984). They do not express MHC class II antigens, but contain numerous

vesicles and form cytoplasmic bridges that separate adjacent lymphocytes in the PP from the intestinal lumen. The "M" cells are responsible for the initial antigen uptake from the intestinal lumen since an extraordinary array of foreign material has been shown to attach to and be taken-up by "M" cells (Trier, 1991; Neutra et al., 1992).

In addition to PP, the small intestine contains a large number of isolated lymphoid follicles which can only be identified microscopically. In structure, these follicles are very similar to PP, the function of lymphocytes found in these tissues has not been studied, but the position and structure of these follicles suggest that they may play a role in antigen trapping and lymphocyte priming.

1.6.2. Mesenteric lymph nodes

MLN lie within the mesentery and drain the lymphatics from intestinal mucosa and PP into the thoracic duct. They have the same structure as peripheral lymph nodes with well developed thymus-dependent area, primary follicles, and germinal centers. MLN develop in parallel with PP with rapid T cell immigration before the appearance of primary follicles and germinal centers. Unlike PP where B cells are abundant, T cells predominate in MLN. T cells with Fc receptors for IgA are relatively common and many B cells in MLN carry surface IgA. Lymphocytes primed in the PP may lodge in MLN for a brief period before continuing their migration to the peripheral circulation and ultimately to the mucosal sites. IgA-producing B cells from MLN repopulate

the mucosal sites and virtually all cells active in immunoglobulin production in MLN are committed to IgA synthesis (McWilliams et al., 1977). MLN are the sites where the intestinal and peripheral immune system come into contact. This is reflected by the finding that both mucosal and peripheral lymphocytes can adhere to high endothelial venules within MLN. Thus, MLN may be regarded as an area where complex cellular interactions take place, which may influence the mucosal as well as the peripheral immune responses.

1.6.3. Lamina propria lymphoid cells

The lamina propria of the small intestine is heavily infiltrated by a variety of lymphoid cells including B lymphocytes, plasma cells, T cells, macrophages and DC, as well as eosinophils, basophils, and mast cells (Targan, 1992). Both T cells and B cells appear to be present in approximately equal numbers. More than 80% of B cells in lamina propria are committed to IgA synthesis, whereas 15 to 20% produce IgM and about 2% produce IgE (Kagnoff, 1987). The striking paucity of IgG-producing B cells in the lamina propria contrasts markedly with extra-intestinal sites where IgG-producing cells are dominant. B cells in the lamina propria are more differentiated than those in the peripheral blood; they have already undergone partial differentiation before arriving in the lamina propria. A large proportion of T cells in the lamina propria bear α/β TCR and most are CD4+

cells. These T cells play an important role in B cell growth and differentiation. A small proportion of T cells express CD8 and function as MHC class I-restricted cytotoxic T cells. About 40% of these CD8+ T cells also express HML-1 antigen ($\alpha E\beta 7$ integrin) which is a characteristic of T cells in the intestinal epithelium (Cerf-Bensussan et al., 1987).

Characterization of lymphoid cells isolated from the lamina propria has identified marked differences in their relative frequency, functional state, and phenotype compared to those in the peripheral blood. Most T cells in the lamina propria express the CD45 RO isotype of CD45, a characteristic of the memory T cell population (Zeitz et al., 1991). The lamina propria is also enriched with T lymphocytes expressing CD25 (the α chain of IL-2 receptor) and CD71 (transferrin receptor), suggesting that these cells are already in a state of activation.

A large number of macrophages are present in the lamina propria. In humans and mice, macrophages from the lamina propria are competent to present antigens *in vitro* (Bland et al., 1991). In contrast, macrophages from the lamina propria of rats are poor APC and are able to suppress the antigen-presenting activity of DC (Pavli et al., 1990). Macrophages in the lamina propria also play an important role in bacterial phagocytosis as illustrated in *Mycobacterium avium* intracellular infection in which the lamina propria macrophages contain large quantities of

undigested or partially digested bacteria (Strom et al., 1983). The lamina propria also contains NK cells and LAK cells (Fiocchi et al., 1985), but their role in intestinal physiology and disease is unknown. Eosinophils and mast cells are relatively more abundant in the lamina propria than other compartments of the small intestine. Mast cells can be stimulated to degranulate by immunologic and non-immunologic stimuli. The inflammatory mediators released from mast cells include histamine, proteases, leukotrienes, prostaglandins, and cytokines. These inflammatory mediators express a broad array of activities and have profound effects on intestinal structure and function such as altered vascular permeability, increased contraction of smooth muscle, increased electrolyte secretion, and recruitment of inflammatory cells. Eosinophils appear to be an important component of the host response to *Helminth* infection. The activity of eosinophils, mast cells, and macrophages in the lamina propria is influenced by cytokines, hormones, and neuroendocrine mediators released in the intestinal microenvironment (Zeitz et al., 1991).

1.6.4. Intraepithelial lymphocytes

Lymphocytes located within the intestinal epithelium are termed intraepithelial lymphocytes (IEL). These cells lie in proximity to the basement membrane that separates the intestinal epithelium from the underlying lamina propria. IEL vary in morphology from typical small lymphocytes to medium-size lymphocytes, many of which have cytoplasmic

granules (Guy-Grand et al., 1993). IEL belong to the T cell lineage as majority of IEL express CD3-TCR complex. In normal adult mice, 75% of IEL express α/β TCR and approximately 20% express γ/δ TCR (Guy-Grand et al., 1991). The majority of IEL express CD8 in the form of either homodimer (CD8 αα) or heterodimer (CD8 αβ). The subset with CD8 αα structure (60%) does not contain CD8 β chain and few of these cells are Thy 1+ (Guy-Grand et al., 1991). This subset is not found in any other location in normal adult mice (Dobbins, 1986). It has been suggested that IEL represent a peculiar cell population that differs from the majority of thymus-derived peripheral T cells (Ernst et al., 1985).

The presence of IEL in athymic nude mice, plus the fact that IEL contain an unusually high percentage of lymphocytes expressing γ/δ TCR, suggest that the intestinal epithelium may be an extra-thymic organ of T cell development (Sollid et al., 1988; Croitoru et al., 1992). Recently, it has been proposed that IEL can be divided into two major cell populations, one is thymo-dependent, thought to originate in the thymus and migrate to PP, and ultimately to the epithelial region. They express the α/β TCR, the heterodimeric form of CD8 molecules (CD8αβ), and the Thy 1 marker. The other is thymo-independent, supposed to develop in the intestinal epithelium. This population has either the γ/δ or the α/β TCR, and lacks the CD8 β chain (Guy-Grand et al., 1991). The maturation of IEL is not affected in α/β TCR

knockout mice. Studies using MHC class I, MHC class II, or both MHC class I and class II deficient mice found no evidence of MHC antigens in shaping the α/β T cell repertoire in the intestinal epithelium (Schleussner et al., 1993).

The function of IEL, particularly those bearing γ/δ TCR, is largely unknown. In germ-free mice, the number of IEL is less than conventional mice, but IEL increase dramatically after intraluminal bacterial colonization, indicating that these cells respond to normal gut flora. Increased IEL bearing γ/δ TCR have been demonstrated in celiac disease, but their role in the pathogenesis of celiac disease remains to be defined (Savilahti et al., 1990). IEL express a number of activities *in vitro* including natural killer, suppressor, and specific cytotoxicity (Ernest et al., 1986; Davies et al., 1981), but the specificity and MHC restriction pattern are unknown. A significant proportion of IEL respond to heat shock proteins expressed by stressed or damaged cells, or bacterial components (Born et al., 1990). IEL also respond to superantigens (Aisenberg et al., 1993). IEL show evidence of activation but few divide *in situ* and lymphoblasts are rarely seen. Recently, IEL have been shown to release IFN-γ and IL-5 that regulate epithelial cell growth and MHC antigen expression (Yamamoto et al., 1993; Nadine Cerf-Bensussan, 1984). The close proximity of IEL and epithelium indicates that the IEL may modulate the structure and function of intestinal epithelium. It is also possible

that IEL may function as the first line of defense against pathogen invasion.

1.7. INTESTINAL EPITHELIUM

The intestinal epithelium is separated from the underlying lamina propria by a thin continuous basement membrane composed of a basal lamina and a layer of glycoprotein ground substance. The epithelium covers intestinal villi and their surrounding crypts and is in direct contact with the luminal contents.

The intestinal epithelium is in a dynamic and spatial balance between proliferation and differentiation along the crypt-villus axis. Anatomically, intestinal epithelium can be divided simply into crypt and villus epithelium. The crypt epithelium contains (1) undifferentiated cells which are actively proliferating and may be seen in mitosis; (2) mucus-secreting goblet cells; (3) a variety of different endocrine epithelial cells; (4) rare caveolated or tuft cells; and (5) in most mammals, the Paneth cells with large secretory granules. The function of crypt epithelium includes epithelial cell renewal, exocrine secretion to the lumen, electrolyte and water secretion, and endocrine secretion both into the lamina propria and into the lumen. The villus epithelium contains (1) a large number of absorptive epithelial cells which are also called enterocytes; (2) goblet cells; (3) a few endocrine cells; (4) rare caveolated cells; and (5) in many species,

particularly in the ileum, occasional cup cells. Additionally, the specialized epithelial cells called "M" cells, overlie the apex of PP in the ileum. The major function of the villus epithelium is absorption of nutrients.

The villus is a functional unit of the small intestine and consists of sheets of enterocytes. Each adult intestinal villus contains 2000 to 7000 epithelial cells depending upon the location along the duodenal to ileal axis and is surrounded by about 6 to 14 flask-shaped crypts of Lieberkuhn (Wright et al., 1982). Each crypt contains 350 to 550 epithelial cells and approximately 150 of these cells, situated in the mid-portion of the crypt, pass through the cell cycle every 12 hours resulting in an average production rate of 300 new cells/day/crypt (Potten et al., 1990). The crypt stem cells are generally characterized by three features: (1) their capacity to undergo asymmetric division, producing one daughter that remains as a stem cell and another daughter that undergoes an irreversible commitment to enter the differentiation pathway; (2) their great potential to proliferate; and (3) their ability to retain a position in a particular environmental niche.

In adult animals, each crypt is monoclonal, a multipotent stem cell anchored near the base of the crypt, gives rise to descendants that proliferate and ultimately yield terminally differentiated, non-proliferating columnar absorptive enterocytes, mucus-producing goblet cells, a

complex population of enteroendocrine cells, and defensin and lysozyme-producing Paneth cells (Winton et al., 1988). Paneth cells complete their differentiation program as they descend to the base of each crypt. In contrast, enterocytes, goblet cells, and enteroendocrine cells undergo differentiation and maturation as they migrate in vertical, coherent bands up adjacent villi. Epithelial cells exit the crypts and move up towards the villus at a remarkable velocity of 0.75 cell diameters per hour. It takes about three days to complete the journey to the apical portion of the villus where about 1400 cells/villus/day subsequently undergo exfoliation into the gut lumen (Potten et al., 1990). When epithelial cells differentiate and move up towards the villus tip, the cells undergo functional maturation leading to the formation of brush border microvilli at the apical surface of epithelial cells. The brush border is rich in enzymes such as alkaline phosphatase, peptidase, oligosaccharidase, and sucrase. These enzymes are responsible for the breakdown of oligosaccharides and oligopeptides and it is in this fashion they are absorbed.

The epithelial cells rest on a basement membrane under which vessels of the microcirculation course through a complex matrix structure. Each epithelial cell is circumferentially wrapped at its apical pole with neighboring cells by a junctional complex. The junctional complex consists of three elements: (1) tight junctions,

(2) intermediate junctions, and (3) spot desmosomes. Such junctional complex, particularly the intercellular tight junctions, seals the space between the epithelial cells and thus, in concert with the apical membrane, separates the underlying tissues from the external world. This "barrier" prevents the free passage of molecules and ions across the paracellular pathway and protects the host from the overwhelming pathogens in the intestinal lumen. Ultra-structurally, the tight junctions are a meshwork of inter-connecting P-face strands and complementary E-face grooves. Freeze-fracture studies have revealed that each strand is composed of a chain of closely associated 10 nanometers intra-membrane particles. Further characterization of tight junctions using biochemical and molecular techniques leads to the identification of several junctional proteins. ZO-1, the first protein identified, is a unique component and directly associated with the formation of tight junctions. More recently, three additional proteins, cingulin, ZO-2, and 7H6, have been added to the list. All these proteins belong to the cytoplasmic "plaque" domain of tight junctions. ZO-2 co-localized with ZO-1, whereas cingulin and 7H6 appear to be further away from the cell membrane and more diffusely organized and loosely associated with tight junctions (Citi, 1993). Recently, cytoskeleton components such as actins have been shown to regulate the epithelial paracellular permeability (Madara, 1987). The structure and

function of such "barrier" depend on the continual proliferation, maturation, and metabolism of epithelial cells and are subject to the regulation of cytokines, growth factors, and extracellular matrix.

The mechanisms that control and co-ordinate epithelial cell proliferation and differentiation have not been well defined. It has been shown that cytokines such as transforming growth factor-α (TGF-α) and TGF-β are actively involved in this process (Barnard et al., 1989; Koyama et al., 1989). TGF-β is expressed as epithelial cells migrate from the crypt to villus tips, suggesting that TGF-β may have a role in cell differentiation. The differentiation process of gut epithelial cells is also influenced by reciprocal permissive and instructive interactions between endodermal, stromal, and mesodermal compartments. The interactions between epithelial cells, extracellular matrix, and mesenchymal cells may preserve the lineage differentiation program despite changes in proliferation or rate of cell migration.

When epithelial cell damage or increased cell loss occurs, both the production of new cells by crypts and their subsequent migration can be dramatically increased resulting in the formation of a "flat mucosa" with decreased absorptive area. The increased production of new epithelial cells is also associated with impaired brush border enzyme activity because the new epithelial cells are pushed to the villus tip without final maturation of these enzymes. The

structure and function of the villi are also highly dependent on the integrity of the basement membrane and underlying connective tissues. Inflammatory insults that harm these elements also have profound effects on epithelial function.

1.8. INTESTINAL MICROFLORA

The enteric flora is derived exclusively from the environment. At birth the alimentary tract is sterile, and animals raised in a germ-free environment harbor no micro-organisms in the intestinal lumen. The stomach and small bowel normally contain relatively small numbers of bacteria. In fact, jejunal cultures fail to identify any bacterial growth in about one third of healthy volunteers. When micro-organisms are present, they are usually lactobacilli and enterococci, gram-positive aerobes or facultative anaerobes. These bacteria are present in the jejunum at concentrations up to 1×10^4 viable organisms per gram of jejunal contents. Coliforms may be transiently present in the jejunum but rarely exceed 1×10^3 per gram of contents and anaerobic bacteria are not found in the proximal small bowel in the healthy host (Toskes et al., 1991).

In the ileum, the concentration of micro-organisms increases to 1×10^5 to 1×10^9 per gram of contents. Enterobacteria including coliforms are regularly found in large numbers in the ileum. Anaerobes, which normally can

not survive in the jejunum, frequently colonize the ileum. The most dramatic change in the enteric microflora occurs across the ileocecal valve. The total number of micro-organisms increases up to one-million fold that is approximately 1 x 10^9 to 1 x 10^{12} per gram of colonic contents. The large bowel flora is dominated by fastidious anaerobic organisms such as bacteroides, lactobacilli, and clostridia (Toskes et al., 1991).

The normal enteric flora plays an important role in intraluminal metabolism of nutrients, drugs, and host-derived constituents such as bile acids, urea, and bilirubin. The enteric flora also influences the architecture and function of the intestinal mucosa. The intestinal villi of germ-free animals are more slender and longer than those of conventionally reared animals, the epithelium is more uniform, and the total surface area is reduced by about 10 to 30% (Savage, 1986; Abrams, 1977). The epithelial renewal is markedly retarded in the absence of enteric flora. The mitotic index of crypt cells is lower in germ-free animals, the generation cycle prolonged, and the pool of proliferating cells is smaller. The transit time of epithelial cells moving along the villus is also prolonged in germ-free animals. Leukocytes, particularly IEL and plasma cells in the lamina propria, and the size and number of PP are all dramatically reduced in germ-free animals (Savage, 1986; Abrams, 1977).

Under the physiologic situation, the type and the number of enteric bacteria are maintained in a delicate balance. Gastric acid and intestinal motility are crucial for limiting microbial overgrowth. Whenever intestinal motility is interrupted, bacterial overgrowth ensues. Interactions between luminal bacteria and the gut lumen represent another important but poorly understood mechanism. Such interactions are complicated and probably include (1) mutual competition for available nutrients, (2) alteration of intraluminal pH redox potential, (3) production of toxic metabolites, (4) bowel synthesis of growth factors, (5) and enzyme-sharing and transfer of antibiotic resistance.

Bacterial overgrowth within the small intestine has been documented in a variety of diseases such as blind loop, stringent loop, stasis, or bacterial overgrowth syndrome (Toskes et al., 1991). Gastrointestinal surgery often leads to bacterial overgrowth in the intestinal tract. Changes in intestinal microflora have been demonstrated after IT (Price et al., 1992). Regardless of the cause, the consequence of bacterial overgrowth is that the microflora of the upper small intestine resembles that of the colon. The increased intraluminal bacterial microflora then competes with the host for ingested nutrients and a complex array of clinical problems ensues. The intraluminal bacterial catabolism,

often with production of toxic metabolites, can directly damage epithelial cells. Patients may develop malabsorption, osteomalacia, or vitamin K deficiency secondary to bacterial overgrowth.

CHAPTER 2. STATEMENT OF HYPOTHESIS

Despite recent advances with immunosuppression, graft rejection continues to be a major barrier to successful clinical IT (Todo et al., 1992b). The factors contributing to the increased frequency and severity of intestinal allograft rejection remain poorly defined. Rejection has been attributed to the abundant lymphoid cells that populate the intestinal graft, but attempts to deplete the lymphoid cells from intestinal grafts prior to transplantation have failed to prevent intestinal rejection (Stangl et al., 1990; Gundlach et al., 1991). It is likely that, in addition to gut lymphoid cells, other components within the intestinal graft may play a critical role in graft rejection.

Intestinal rejection is characterized by gut barrier breakdown and bacterial translocation (McAlister et al., 1993a). It has been shown that animals with rejecting intestinal allografts have large numbers of aerobic enteric bacteria in their liver, spleen, and MLN (Grant et al., 1991a). We hypothesized that bacterial translocation may result in the release of endotoxin, the lipopolysaccharide (LPS) components of gram-negative bacterial cell walls, which amplifies the rejection process by stimulating the production of inflammatory cytokines such as IL-1, IL-6, and TNF-α as well as upregulating the surface expression of adhesion molecules. The intestinal epithelial cells may be particularly vulnerable to inflammatory insult since they

are directly exposed to the luminal bacteria and LPS, and are in close proximity to cytokine-producing cells as well as in a state of rapid proliferation and differentiation along the crypt-villus axis. Intestinal epithelial cells may also play an important role in regulating leukocyte migration at the mucosal level through expression of adhesion molecules.

For my Ph.D. thesis, endotoxin and inflammatory cytokines (IL-1, IL-6, TNF-α) during acute intestinal rejection were examined and their effects on intestinal epithelial cells were further characterized. This project involved the development of several models, briefly summarized below.

1. The clinical and histological features of acute intestinal rejection were examined in a novel mouse model of IT. Endotoxin, IL-1, IL-6, and TNF-α in the peripheral blood during acute intestinal rejection were determined.

2. An epithelial cell clone (IEC-4.1) from the small intestine of BALB/c mice was established by SV40 DNA transfection and further characterized.

3. The cytotoxic effects of endotoxin and inflammatory cytokines on IEC-4.1 cells were examined *in vitro*.

4. Inflammatory Cytokine (IL-1, IL-6, TNF-α, TGF-β) and adhesion molecule (ICAM-1, VCAM-1) gene expression in IEC-4.1 cells were analyzed using northern blot analysis.

5. The molecular basis of macrophage adhesion to IEC-4.1 monolayers was also examined.

CHAPTER 3. ENDOTOXIN AND INFLAMMATORY CYTOKINES IN ACUTE INTESTINAL REJECTION IN THE MOUSE

3.1. INTRODUCTION

The intestine is continuously exposed to an environment of anaerobic and gram-negative bacteria. The breakdown of gut barrier function, followed by bacterial translocation has been demonstrated during acute intestinal rejection (Grant et al., 1991a). We hypothesized that bacterial translocation may result in the release of endotoxin, the LPS components in the outer cell membrane of gram-negative bacteria, that amplifies the graft rejection process by stimulating the production of inflammatory cytokines. Endotoxin is a potent chemoattractant to inflammatory cells and stimulates immunocytes, particularly macrophages, to produce IL-1, IL-6, and TNF-α that are capable of inducing local tissue damage through their actions on T cells, inflammatory leukocytes, and blood vessel endothelial cells (Nestel et al., 1992; Pace et al., 1981). Previous studies have shown that the intestinal tract is a primary source of endotoxin in patients with multiorgan failure, GVHD, malnutrition, and trauma in which the gut barrier function is often compromised (Hart et al., 1979; Caridis et al., 1972; Cuevas et al., 1974). Increased gut permeability and bacterial translocation have also been shown in active inflammatory bowel disease and inflammatory cytokines are

the important mediators of mucosal damage (Targan et al., 1991).

Many of the toxic effects of endotoxin can be mimicked by TNF-α and IL-1, which synergize with each other (Welbourn et al., 1992). Indeed, mice in which the gene for the TNF-α receptor, TNFRp55, has been disrupted are resistant to endotoxin shock (Pfeffer et al., 1993). Furthermore, pretreatment of mice with antibodies against TNF-α prevents death induced by the injection of endotoxin (Mannel et al., 1987; Old, 1987), and rabbits can be protected from septic shock by the injection of an IL-1 receptor antagonist shortly before the injection of endotoxin (Ohlsson et al., 1990).

The role of endotoxin and inflammatory cytokines in the pathogenesis of intestinal rejection, particularly in epithelial damage, has not been investigated in any detail. In this study, we examined endotoxin and temporal expression of inflammatory cytokines in acute intestinal rejection in the mouse.

3.2. MATERIALS AND METHODS

3.2.1. Animals

Male BALB/c ($H\text{-}2^d$) and (C57BL/6 x BALB/c)F1 ($H\text{-}2^{b/d}$) inbred mice weighing 25 to 30 grams were purchased from Harlan Sprague-Dawley Incorporation (Indianapolis, IN, USA). The animals were housed in the John P. Robarts Research Institute (London, Ontario, Canada) in accordance with

guidelines established by the Canadian Council on Animal Care (1984).

3.2.2. Surgical model

The surgical model of IT in mice developed by Dr. Robert Zhong and Dr. Zheng Zhang (1993) in the Microsurgery laboratory in the John P. Robarts Research Institute, London, Ontario is illustrated in Figure 1 and described in detail below.

Preoperative care and anesthesia: The donor animals received 5% glucose and 0.9% saline orally *ad libitum* for 6 hours before surgery. Food and water were not restricted in the recipients. Atropine (0.04 mg/kg) was given by subcutaneous injection to the donors and the recipients before anesthesia. Intramuscular cefoxitin (40 mg/kg) was administered to the recipients. The mice were anesthetized with an intraperitoneal injection of pentobarbital (65 mg/kg).

Donor operation: The donor procedure was modified from our rat IT model (Zhong et al., 1991). The abdomen was entered via a midline incision. A wet cotton swab was used when necessary to handle the graft. Direct manipulation of the intestine was minimized to avoid graft damage. The jejunum and ileum were identified and the mesenteric vessels were ligated with 8-0 silk sutures. The entire jejunum and ileum were resected. The portal vein was carefully separated from the pancreas. Following exposure of the aorta, the renal arteries and celiac trunk were ligated. The pyloric

and splenic veins were ligated separately using 8-0 silk sutures. A 30-gauge needle was then introduced into the aorta and the graft was perfused *in situ* with 0.2 to 0.4 ml of cold, heparinized Ringer's lactate solution. After dividing the portal vein close to the hilum of the liver, the graft was removed with a Carrel patch of aorta and stored in lactated Ringer's solution at 4°C. The donor surgery took about 45 minutes and 0.4 to 0.6 ml of normal saline was given intravenously during the procedure.

Recipient operation: The recipient's intrarenal aorta and inferior vena cava were carefully mobilized and isolated using microvascular clamps. An elliptical aortotomy was made by gently grasping the adventitia of the aorta and lifting it vertically with jewelers forceps. A pair of iris scissors was used to make a single cut at an angle of 30° from the most distal part of the aorta. This cut was approximately one-fifth the diameter of the vessel. Care was taken to avoid a larger aortotomy, which would inevitably cause a narrowing of the anastomotic site.

Venous anastomosis: Just above the aortotomy, a longitudinal venotomy was made in the inferior vena cava by puncture with a 30-gauge needle. Two 11-0 nylon stay sutures (Shapoint, Reading, PA, USA) were placed at both apices of the venotomy. The donor small intestine was then removed from the ice, and placed in the right side of the mouse. After ensuring that the donor portal vein was not twisted, an end-to-side anastomosis was performed using a continuous

11-0 nylon suture. The posterior wall was anastomosed from within the vessel lumen without repositioning the graft. The anterior wall of the portal vein was anastomosed externally using the same suture. Saline irrigation was used to keep the vessel walls apart during the anastomosis. Before tying the sutures, the vein was gently pulled apart to avoid collapse and narrowing of the vessel at the anastomotic site.

Arterial anastomosis: For arterial anastomosis, the front and back walls of the artery were joined with continuous sutures. After completion of the front wall, the intestinal graft was rotated to the left to facilitate suturing of the back wall of the artery. The graft was rinsed with cold saline several times during the anastomosis. A small quantity of microfibrillar collagen (Avitene®) was placed around the arterial anastomosis before releasing the clamps. Gentle pressure was applied to the anastomosis with a dry cotton swab for 1 to 2 minutes after revascularization. Five units of heparin in saline were given intravenously immediately after graft reperfusion.

Graft exteriorization: The native intestine was left intact. Both ends of the graft were exteriorized as stomas. The stomas were secured with two 9-0 nylon sutures between the host peritoneum and the seromuscular layer of the graft, and three 7-0 silk sutures between the skin and the everted

mucosa of the graft. The abdomen was closed in a single layer with a continuous 5-0 silk suture.

Postoperative care: The recipient received a total of 0.8 ml of normal saline by intermittent injection in the dorsal penile vein during the procedure, including 0.3 ml of saline given before and after vascular clamping. The rate of infusion was very slow to avoid congestive heart failure due to sudden fluid overload. After closing the abdomen, 1.5 ml of saline was given subcutaneously. Blood transfusions were not necessary. Postoperatively, the mice were kept on a warm blanket and under a heat lamp for the first 24 hours. They usually recovered from anesthesia within 1 hour after the operation. The mice were given regular water and food *ad libitum*. Thirty units of heparin were given subcutaneously twice daily for 24 hours.

Two groups of transplants were performed: (1) allografts in which (BALB/c x C57B6) F1 intestinal grafts were transplanted into BALB/c recipients, with no immunosuppressive drugs being given; and (2) isograft control group in which BALB/c intestinal grafts were transplanted into BALB/c mice. The use of F1 mice as graft donors eliminated the possible effect of GVHD and any change observed was due to rejection. Three animals in the allograft group were sacrificed on post operative day (POD) 3, 5, 7 and 10 (POD 3, 5, and 7 for the isograft controls) for histologic and immunologic studies.

3.2.3. Histology

At necropsy or sacrifice, one centimeter tissue segments from the distal end of the intestinal grafts were dissected and immediately fixed in 10% phosphate buffered formalin (pH 7.4) at room temperature overnight. The tissue specimens were embedded in paraffin and cut into 3 um sections. The tissue sections were mounted onto glass slides and stained with hematoxylin and eosin. The graft histology was examined by light microscopy by a pathologist under blinded conditions. Particular attention was paid to pathologic changes within the lamina propria, epithelium, and villus architecture for the following features: mitosis, cryptitis, loss of goblet cells, lymphocytic infiltration, and sloughing of villus tips. The histology of intestinal mucosa was graded from 0 to 2. 0 was defined as no change compared to the isograft control; 1 was characterized as minimal change; and 2 was defined as marked change.

3.2.4. Serum sample preparation

Whole blood was obtained at sacrifice by cardiac puncture. The recipients were anaesthetized with ether and the chest was sterilized with 75% ethanol. A 22 gauge needle was introduced into the heart and approximately 1 ml of blood sample was collected in a 5 ml syringe. The blood sample was transferred to a sterilized Eppendorf tube (DiaMed Lab Supplies, Mississauga, Ontario, Canada) and kept at 4°C overnight without anti-coagulation treatment. The

serum, separated by centrifugation at 4000 rpm for 20 minutes at 4°C, was stored at -70°C.

3.2.5. Limulus amoebocyte lysate assay

Endotoxin was determined using a chromogenic limulus amoebocyte lysate assay (Levin et al., 1970). The limulus amoebocyte lysate kit which contains pyrogen-free water, LPS standard control (*Escherichia coli*, 0111:B4), chromogenic substrate, and limulus amoebocyte lysate, was purchased from Biowhittaker Inc. (Walkersville, MD, USA). The serum samples obtained from animals at sacrifice were diluted 1:10 with pyrogen-free water and incubated in a water bath at 70°C for 5 minutes to inactivate non-specific inhibitors in the serum. Serial dilutions of serum samples along with the LPS standard control were prepared in 96-well, flat-bottom tissue culture plates (50 ul/well) (Flow Laboratories, McLean, Virginia, USA), followed by addition of 50 ul of limulus amoebocyte lysate to each well. The plates were incubated at 37°C for 20 minutes and then the colorimetric substrate solution was added (100 ul/well). The plates were incubated for an additional 20 minutes and the reaction was terminated by addition of 10% SDS solution. During the incubation, endotoxin converts the proenzyme in limulus amoebocyte lysate to enzyme which in turn catalyzes a synthetic substrate to a yellow product called p-nitroaniline. The optical density (OD) was quantitated using a ELISA plate reader (TiterTek Multiskan Plus, Mississauga, Ontario, Canada) with a 410 nm filter. The

absorbance at 405 to 410 nm is in direct proportion to the amount of endotoxin present. The result was expressed as the mean OD value ± standard deviation (SD) of 3 animals in each group.

3.2.6. Cytokines and cell lines

Human recombinant IL-1α (1000 U/ml), IL-1β (1000 U/ml), and IL-6 (20,000 U/ml) were purchased from Bioproducts for Science Incorporation (Indianapolis, IN, USA). Human recombinant TNF-α (0.5 mg/ml) was obtained from Genentech Incorporation (San Francisco, CA, USA).

L929, a TNF-α sensitive fibroblast cell line, was obtained from American Type Culture Collection (ATCC, Rockville, Maryland, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Burlington, Ontario, Canada) supplemented with 10% fetal calf serum (FCS) (Sigma, St. Louis, MO, USA), 100 U/ml penicillin and streptomycin, 1% non-essential amino acid, 1 mM sodium pyruvate, and 2 mM L-glutamine (Gibco BRL).

LBRM-TG6 (from ATCC), an IL-1 sensitive cell line, was cultured in Iscove's modified Eagle's medium (IMEM) (Gibco BRL) containing 10% FCS, 100 U/ml penicillin and streptomycin, and 2×10^{-5} 2-mercaptoethnol.

B9 (from ATCC), an IL-6 dependent cell line, was cultured in IMEM containing 5% FCS, 100 U/ml penicillin-streptomycin, 2×10^{-5} 2-mercaptoethnol, and 100 U/ml human recombinant IL-6. CTLL-2, an IL-2 dependent cell line, was kindly provided by Dr. B. Singh (Department of Microbiology

and Immunology, University of Western Ontario, London, Ontario, Canada).

CTLL-2 cells were cultured in RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin-streptomycin, and 10% supernatant of Concanavalin A-stimulated rat spleen cells.

3.2.7. Cytokine bioassays

IL-1 was assayed using the LBRM-TG6 cell line. LBRM-TG6 cells release IL-2 in the presence of IL-1 along with a sub-stimulatory dosage of phytohemagglutinin (L-8754, Sigma), which supports the proliferation of CTLL-2 cells (Larrick et al., 1985). Serial dilutions of serum samples or IL-1 standard control (50 ul/well) were prepared in 96-well, flat-bottom tissue culture plates (Flow Laboratories) with IMEM. LBRM-TG6 cells were treated with Mitomycin C (M-0503, Sigma) at 25 ng/ml at 37°C for 30 minutes to stop the cell proliferation and then added into the culture plates (2×10^4 cells/well) along with phytohemagglutinin (3 ug/ml). The culture plates were incubated at 37°C for 24 hours, followed by the addition of CTLL-2 cells (1×10^4 cells/well). The cell mixture was incubated for another 24 hours. Proliferation of CTLL-2 cells was measured using a colorimetric assay (Mosmann et al., 1989).

The colorimetric kit that contains a tetrazolium dye solution and a solubilization solution was purchased from Promega (Madison, WI, USA). After tetrazolium dye solution (15 ul/well) was added, the plates were incubated at 37°C

for 4 hours, during which time the living cells convert the tetrazolium salt into a blue insoluble formazan product. A solubilization solution (100 ul/well) was then added to each well to solubilize the formazan and produce a colored solution. The OD of each well was read using a ELISA plate reader (TiterTek Multiskan Plus) with a 590 nm filter.

IL-6 activity was determined using the B9 cell line (Tono et al., 1992). Briefly, serial dilutions of serum samples or IL-6 standard control were prepared in IMEM. B9 cells (1×10^4 cells/well) were cultured for 3 days at 37°C with serum sample and IL-6 standard dilutions in 96-well plates (Flow Laboratories) and the cell proliferation was determined using the colorimetric assay (Mosmann et al., 1989).

TNF-α activity was measured using the L929 cell line. The cells were diluted to 1×10^5 cells/ml, then plated into 96-well culture plates (Flow Laboratories) at 100 ul/well. The plates were incubated at 37°C for 24 hours. Before the addition of serum samples, the cells were briefly treated with actinomycin D (A-1410, Sigma) at a final concentration of 1 ug/ml for 2 hours, then the serially diluted serum samples or TNF-α control (100 ul/well) were added and cultured for 18 hours. Cell death was determined using the colorimetric assay (Mosmann et al., 1989). The OD value of microplate wells containing medium alone was used as the baseline. The OD value of each well subtracted from the baseline OD represents the TNF-α activity in the serum.

3.2.8. Statistics

The data are expressed as mean OD ± SD of 3 animals in each group and compared using the analysis of variance with a p value less than 0.05 as the level of significance.

3.3. RESULTS

3.3.1. Clinical course of acute intestinal rejection

Animals that died within 24 hours of surgery were considered surgical failures (10 to 15%) and were excluded from this study. The remaining mice were active and had normal-appearing stomas during the first 3 days after surgery. Mice with allografts had a marked increase in the output of mucus from stomas on POD 6 (± 0.3 days) and obliteration and stenosis of the stoma on POD 8 (± 0.5 days). A palpable abdominal mass developed on POD 10 (± 0.3 days). At necropsy, mice with intestinal allograft had the following findings: (1) necrotic and ruptured stomas; (2) purulent fluid in the peritoneum; (3) caseous fluid within the intestinal graft lumen; and (4) multiple liver abscesses. The isograft recipients remained well with normal-appearing stomas until sacrifice.

3.3.2. Histology of acute intestinal rejection

Table 1 summarizes the histological changes in the intestinal grafts at necropsy. In the intestinal allograft group, epithelial alterations were one of the prominent features of acute intestinal rejection. Histologically, two distinct phases of epithelial damage have been observed. A

proliferative phase that developed around POD 7 was characterized by increased epithelial turnover, crypt hypertrophy, and crypt cell hyperplasia. The epithelial architecture was generally normal and epithelial necrosis was not seen at this stage (Plate 1). A destructive phase that presented around POD 10 was manifested by villus atrophy, crypt necrosis, and epithelial sloughing (Plate 2). In most cases, bacterial attachment to the epithelium was observed. The increased crypt cell proliferation occurred prior to villus atrophy and this process was always associated with pericryptal cellular infiltrates. The isografts had normal intestinal histology at all time points.

3.3.3. Circulating endotoxin in acute intestinal rejection

Endotoxin was detected in the serum of both intestinal allograft and isograft recipients as early as on POD 3, although the endotoxin level in the isograft group was lower than that in the allograft group, suggesting that the early endotoxemia after IT was probably due to the non-specific injury to the intestinal epithelium during surgery. In the intestinal isograft recipients, the serum endotoxin progressively decreased thereafter and remained undetectable from POD 5. In the intestinal allograft recipients, however, the serum endotoxin slightly decreased on POD 5 and significantly elevated on POD 7 and POD 10 ($p<0.05$) (Figure 2).

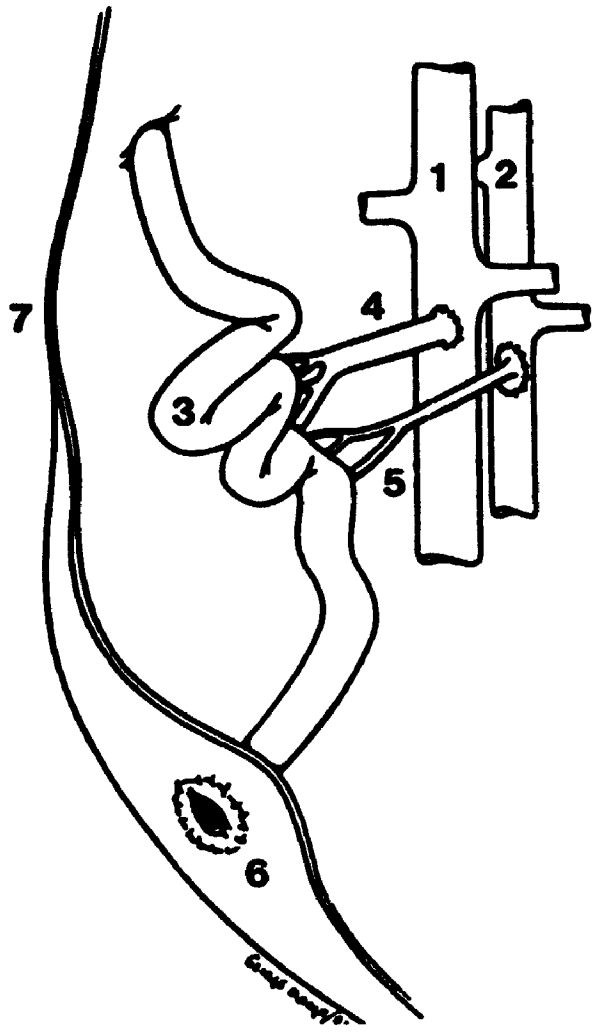
3.3.4. Inflammatory cytokines in acute intestinal rejection

Significant levels of IL-1 activity were not detected in the peripheral blood of both intestinal allograft and isograft recipients at any time point (Figure 3).

IL-6 was readily detectable in the peripheral blood of both intestinal allograft and isograft recipients on POD 3 compared to the medium control ($p<0.05$). In the intestinal isograft group, IL-6 activity progressively decreased and reached the baseline level by POD 5. In contrast, IL-6 activity significantly increased in the intestinal allograft group on POD 5 and peaked on POD 7 ($p<0.05$) (Figure 4).

TNF-α activity was not detected in the peripheral blood of intestinal allograft recipients until POD 5. TNF-α activity progressively increased and remained at a high level on POD 10. TNF-α activity was not detected in the isograft control group at any time point (Figure 5).

Figure 1. Surgical model of IT in the mouse



1: Recipient inferior vena cava; 2: Recipient aorta; 3: Intestinal graft; 4: Graft portal vein; 5: Graft superior mesenteric artery and aortic patch; 6: Distal stoma; 7: Abdominal wall.

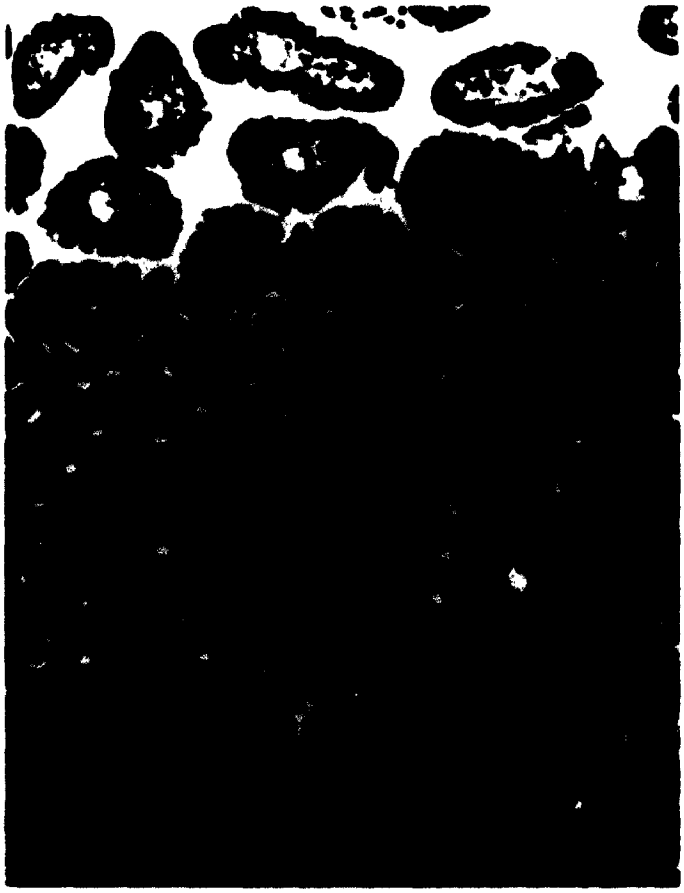
Table 1. Median histological scores of changes in intestinal allografts

	POD 3	POD5	POD 7	POD 10
Mitoses	0.5	1.5	1.5	N/A
Cryptitis	0.5	1.0	1.5	N/A
Goblet cell loss	0	2.0	2.0	2.0
Lymphocytic infiltration	0	0	1.0	1.0
Villus tip sloughing	0	0	0	1.0
Epithelial erosions	0	0	0	1.0

N/A not available

The histology of intestinal mucosa was graded from 0 to 2 for the following features: mitosis, cryptitis, loss of goblet cells, lymphocytic infiltration, and sloughing of villus tips. 0 was defined as no change compared to the isograft control; 1 was characterized as minimal change; and 2 was defined as marked change. All of the isografts had normal histology.

Plate 1. The proliferative phase of epithelial damage in acute intestinal rejection

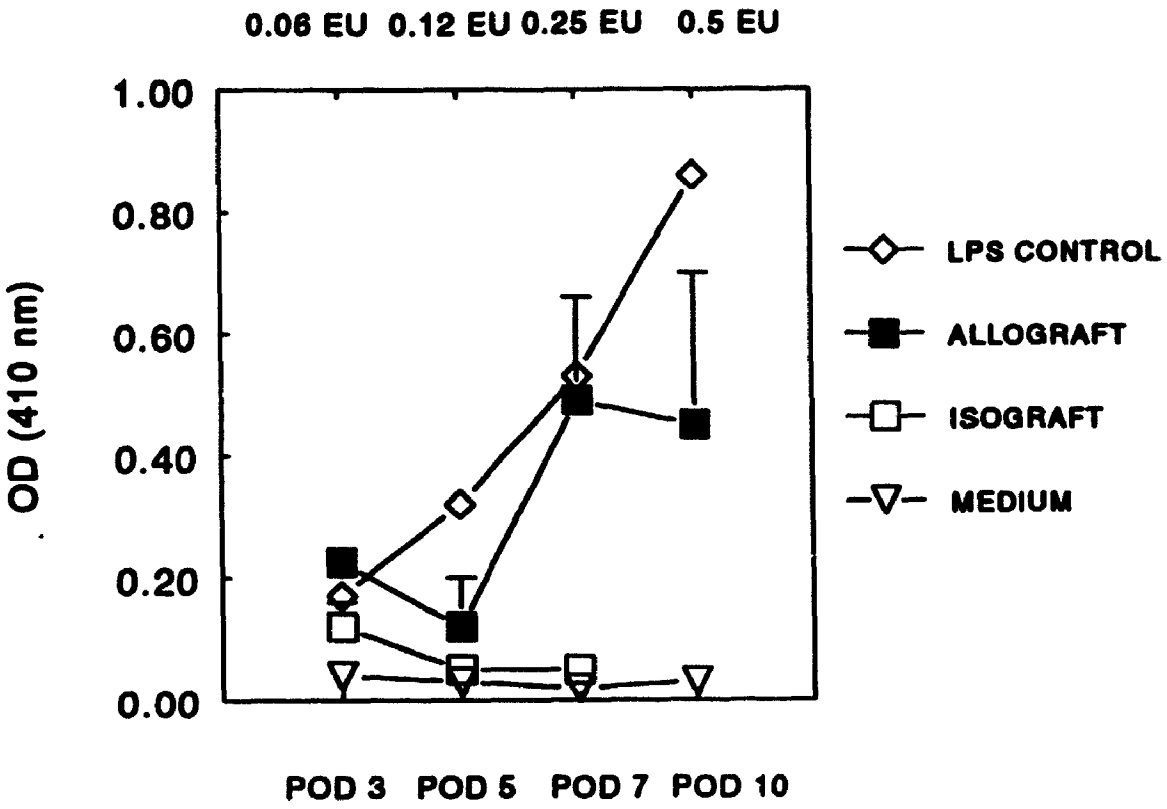


The histologic changes of intestinal allograft on POD 7. This picture shows villus atrophy, crypt hypertrophy, and crypt cell hyperplasia with lymphocytic infiltration, but the general architecture of intestinal epithelium was normal. 150 X.

Plate 2. The destructive phase of epithelial damage in acute intestinal rejection

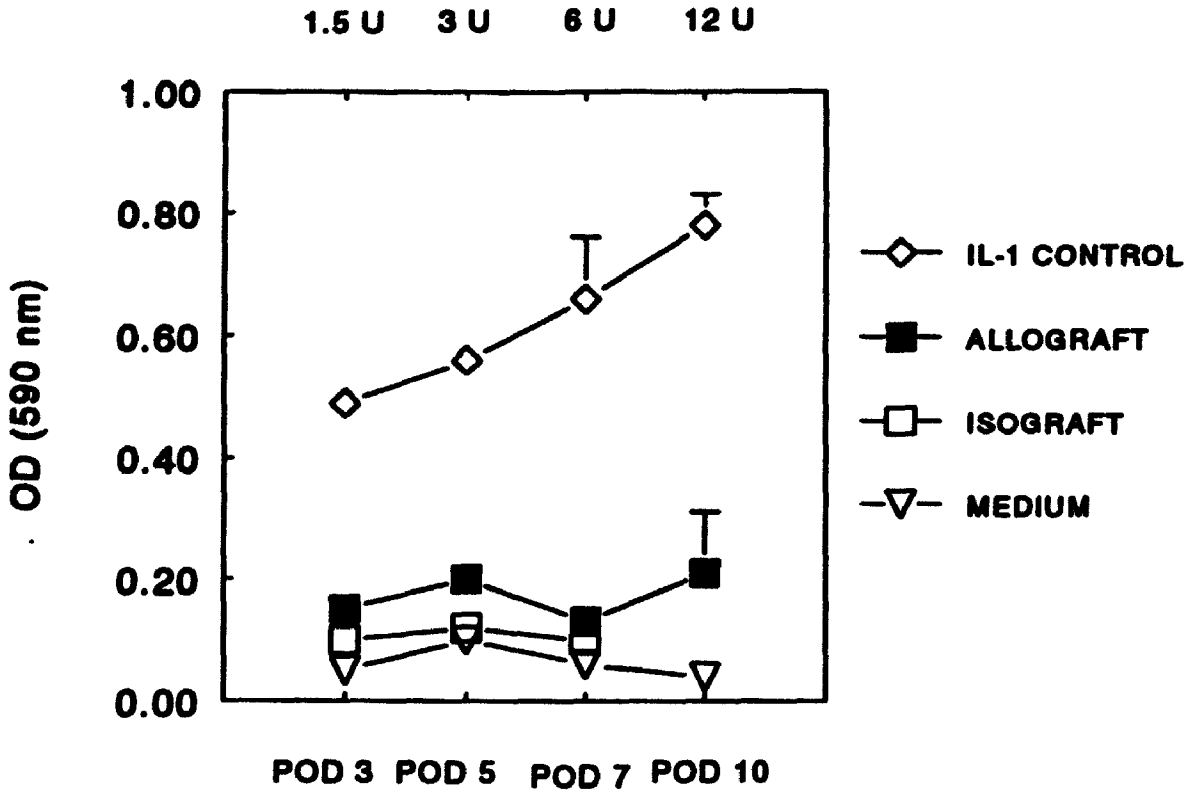
The histologic changes of intestinal allograft on POD 10. This picture shows epithelial sloughing, crypt necrosis, and heavy cellular infiltrate in the intestinal epithelium, bacterial attachment to the luminal surface was often present at this time. 150 X.

Figure 2. Serum endotoxin level during acute intestinal rejection



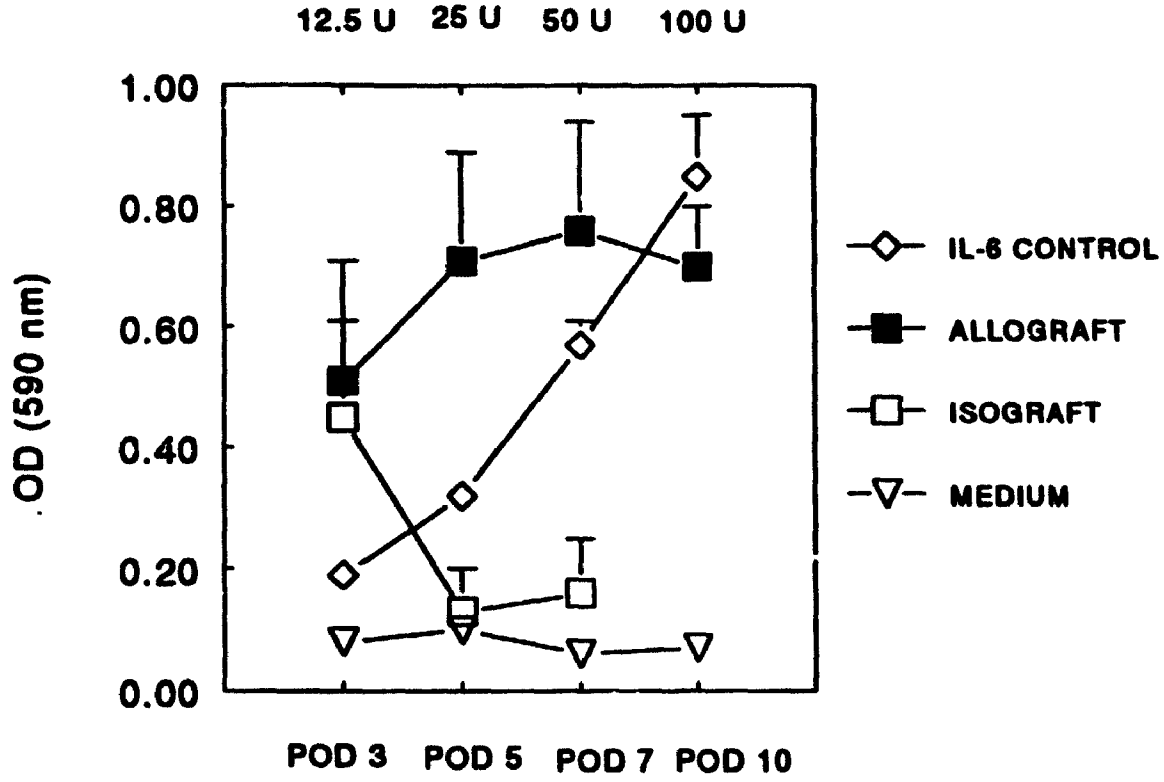
Serum from mice receiving intestinal isografts or allografts was diluted 1:10 with pyrogen-free water and incubated at 75°C for 5 minutes to inactivate the non-specific inhibitors in the serum. The endotoxin level in the serum was assayed using chromogenic limulus assay. The Y axis is the OD value at 410 nm. The top X axis is the LPS control shown in EU/ml. Each point in the Figure represents the mean OD $\pm$ SD of 3 animals in each group.

Figure 3. Serum IL-1 level during acute intestinal rejection



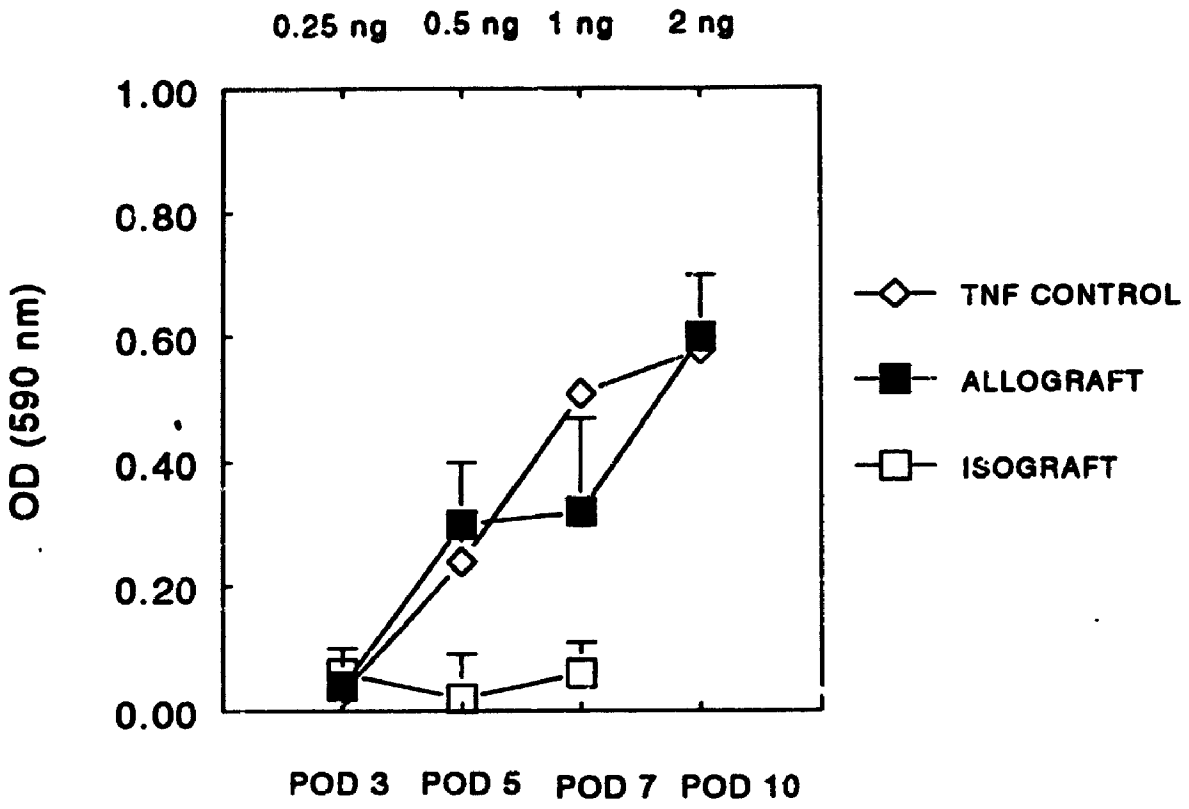
Serum was diluted 1:2 with IMEM medium and assayed for IL-1 activity using the LBRM-TG6 cell line. LBRM-TG6 cells release IL-2 in the presence of IL-1 and sub-stimulatory dosage of PHA, which support the proliferation of CTLL-2 cells. The proliferation of CTLL-2 cells was quantitated by the colorimetric assay. Cells cultured with medium alone were included in each assay as the negative control. The Y axis is the OD value at 590 nm. The top X axis is the IL-1 control shown in U/ml. Each point in the Figure represents the mean OD $\pm$ SD of 3 animals in each group.

Figure 4. Serum IL-6 level during acute intestinal rejection



Serum was diluted 1:2 with IMEM and assayed for IL-6 activity using the B9 cell line. The proliferation of B9 cells was determined by the colorimetric assay. B9 cells cultured with medium alone were included as the negative control. The *f* axis is the OD value at 590 nm. The top X axis is the IL-6 control shown in U/ml. Each point in the figure represents the mean OD $\pm$ SD of 3 animals in each group.

Figure 5. Serum TNF-α level during acute intestinal rejection



Serum was diluted 1:2 with DMEM and assayed for TNF-α activity using the L929 cell line. The killing of L929 cells was quantitated by the colorimetric assay. The Y axis is the OD value at 590 nm. The top X axis is the TNF-α control shown in ng/ml. Cells cultured with medium alone were included in each assay as the baseline control. The OD value of each well, subtracted from the baseline OD value, represents the TNF-α activity in the serum. Each point in the Figure represents the mean OD ± SD of [illegible] animals in each group.

3.4. DISCUSSION

This study demonstrates that acute intestinal rejection is associated with elevated levels of endotoxin, IL-6, and TNF-α in the peripheral blood. IL-6 was elevated prior to histological changes of graft rejection while the progressive increase of endotoxin and TNF-α activity was correlated with the histological severity of epithelial damage.

Several factors may influence the circulating level of endotoxin during acute intestinal rejection. The integrity of the gut barrier is compromised during IT leading to increased gut permeability. There are several pathways whereby the gut barrier function can be damaged. Graft preservation, cold ischemia, and reperfusion injury during surgery can damage the intestinal epithelium since there was a transient endotoxemia as early as on POD 3 in the intestinal isograft control group, but this damage is transient and reversible. The intestinal epithelium can recover from such injury in the absence of additional insults. In the intestinal allograft recipients, however, the rejection process overtakes the epithelial recovery and induces progressive epithelial alterations and this process is accompanied by increased gut permeability (Grant et al., 1991a). The pathogenesis of epithelial damage and gut barrier breakdown during graft rejection is largely unknown. Experimental evidence suggests that the products of activated T cells influence the structure and function of

epithelial junctional complex (Madara et al., 1990; O'Farrelly et al., 1992; Madara et al., 1989). The transplantation process causes decreased gut motility and results in bacterial overgrowth (Price et al., 1992). Bacterial overgrowth, therefore, increases the frequency and severity of bacterial translocation and endotoxin release (Browne et al., 1991). Furthermore, the luminal bacteria-derived endotoxin and the bacterial metabolites can directly damage the junctional complex between epithelial cells (Deitch et al., 1991; Barber et al., 1991). The level of endotoxin in the peripheral circulation is also affected by whether the intestinal allograft is drained into the systemic circulation or into the portal circulation. The liver is an efficient scavenger of endotoxin in portal blood (Decker, 1990); this substance is normally only released into the peripheral circulation when the liver is saturated or severely diseased. Clearance of endotoxin by the native liver may be one of the reasons that allogeneic rat intestinal allografts that are drained into the portal vein survive longer than grafts that are drained into the vena cava (Schraut et al., 1985).

Concurrent with the increased levels of endotoxin, IL-6 and TNF-α were also elevated in the peripheral blood during acute intestinal rejection. IL-6, derived mainly from macrophages and monocytes, is important in graft rejection since it promotes the growth and differentiation of both T cells and B cells. IL-6 also regulates acute phase response

and costimulates T cell activation (Hirano et al., 1990). IL-6 has been shown to interfere with the formation of epithelial tight junctions and therefore may contribute to the increased gut permeability (Madara et al., 1989).

The production of TNF-α clearly plays an important role in epithelial damage during intestinal rejection since the progressive upregulation of this cytokine correlated well with the histological severity of epithelial damage. Previous studies have shown that systemic administration of TNF-α to mice causes small intestinal damage, characterized by crypt hypertrophy, villus atrophy, focal necrosis, and hemorrhage (Sun et al., 1988; Garside et al., 1993), which are also key histological features of intestinal rejection observed in this study. The expression of TNF-α has also been noted with lung rejection (Cheung et al., 1993; Saito et al., 1993). This cytokine may play a particularly important role in rejection of the solid organ transplants that contain mucosal interfaces which are in contact with microflora.

In contrast to IL-6 and TNF-α, IL-1 activity was not detected in the peripheral blood although endotoxin is also a potent inducer of IL-1 production. This cytokine may be elaborated locally in the intestinal graft and the reticuloendothelial system since IL-1 mRNA was progressively upregulated during acute intestinal rejection in the mouse (Quan et al., 1993). It is also possible that the release of this cytokine involves post-transcriptional and

post-translational regulation (Durum et al., 1985). The absence of a signal peptide on IL-1 causes much of the synthesized cytokine remains within cells or membrane bound (Dinarello, 1988), and therefore, it may be difficult to detect in the peripheral circulation. The fact that this cytokine was not detected in the serum may also reflect limitations in the sensitivity of the bioassay used in this study.

This study suggests that the primary target of endotoxin-mediated damage may be the intestinal epithelium. Endotoxin, in conjunction with the inflammatory cytokines, may cause profound epithelial destruction, either directly or indirectly through the activation of other inflammatory cells. This is supported by the histological studies showing that transplantation of fetal mouse intestine free of enteric bacteria under the kidney capsule, the rejection process is characterized by crypt hypertrophy and progressive villus atrophy, but epithelial cells are normal, and mucosal plasma cells and leukocyte infiltrate are absent (MacDonald et al., 1992b). Furthermore, the crypt necrosis is absent in the rejection of germ-free intestinal allografts implanted under the kidney capsule (MacDonald et al., 1977; Ferguson et al., 1977).

The precise source of inflammatory cytokines during acute intestinal rejection was not directly addressed in this study. TNF-α is produced by T cells in response to allogeneic stimulation and also by macrophages in response

to endotoxin. Although IL-6 is predominately released from macrophages stimulated by endotoxin, other cell types also produce IL-6. Previous studies have shown that elevated serum levels of TNF-α were found in renal allograft rejection in humans (Van-Oers et al., 1988; Maury et al., 1987) and increased IL-6 activity was also demonstrated in the serum and bile after liver transplantation in rats (Tono et al., 1992). In renal transplantation in humans, the increased TNF-α activity occurred early and appeared to be transient (Maury et al., 1987). In acute liver allograft rejection in rats, the elevated IL-6 activity peaked at 2 days after transplantation and then declined thereafter, IL-6 activity was comparable to the isograft control by POD 7 (Tono et al., 1992). In the present study, however, the temporal pattern of cytokine activity showed different characteristics. IL-6 and TNF-α activity were progressively increased in the peripheral blood and correlated with the high levels of endotoxin in the blood, suggesting that endotoxin is an important stimulator of prolonged TNF-α production in acute intestinal rejection.

The relationship between graft rejection and endotoxemia in acute intestinal rejection is probably bi-directional. The initial T cell activation initiated by alloantigens results in the release of cytokines that damage the epithelial barrier function leading to increased gut permeability. The increased gut permeability along with bacterial overgrowth then results in bacterial translocation

and endotoxin release. Endotoxin may, in turn, amplify the rejection process by activating macrophages and inducing the production of inflammatory cytokines. It seems more likely that bacterial products influence the level of immune responsiveness and serve as a secondary amplification process to cause accelerated epithelial damage.

Further studies are warranted to examine the precise source of inflammatory cytokines, their role in directing cellular infiltration in the intestinal epithelium as well as their role in epithelial damage by using selected intestinal tissue. To further define the source of inflammatory cytokines and the role of epithelial cells in intestinal rejection, we established an epithelial cell clone from the small intestine of BALB/c mice and further addressed these questions as described in the following chapters.

CHAPTER 4. ESTABLISHMENT AND CHARACTERIZATION OF INTESTINAL EPITHELIAL CELL LINES FROM MOUSE SMALL INTESTINE

4.1. INTRODUCTION

The pathogenesis of epithelial damage during acute intestinal rejection is not well defined. Further characterization of this important cell type has been hampered by the lack of successful methods to culture small intestine-derived epithelial cells *in vitro* because of their limited proliferative capacity and the overgrowth of other fast-growing cell types such as fibroblasts and smooth muscle cells.

There are a few reports on the establishment of epithelial cell lines, such as T84, CaCo-2, and HT-29, from human colonic carcinomas. Some epithelial cell lines have been established from the small intestine of fetal rats (Quaroni, 1994). Although these cell lines have been widely used for the study of epithelial cell physiology, all have their limitations (McKay et al., 1993). For example, T84 cell line has a secretory phenotype analogous to the intestinal crypt cells. It forms tight monolayers that display extremely high electrical resistance. CaCo-2 cells develop a low spontaneous potential difference and display a chloride conductance similar to intestinal secretory cells. HT-29 cells express receptors for vasoactive peptides, neurotransmitters, prostaglandins, and catecholamines, and

thus are likely to be a valuable model to study the neuroimmune regulation of epithelial activity. None of these cell lines constitutively expresses MHC class II molecules.

There have been many attempts to immortalize epithelial cells using viruses and oncogenes. The introduction of SV40 virus into human colonic epithelial cells was described in the early 1980s, although cells continued to die after only a few passages (Moyer et al., 1984). The early region protein gene of adenovirus (E1A) has been used to immortalize fetal rat intestinal epithelial cells. An attempt was also made to immortalize epithelial cells with polyoma virus and the SV40 large T gene under the control of the heavy metal inducible metallothionein promoter (Paul et al., 1993). However, the epithelial cells were not stably immortalized by these agents and gradually died in culture after a number of months (Emami et al., 1989). Recently, a temperature-sensitive mutant of the SV40 large T gene has been used to produce a transgenic mouse strain (Hauft et al., 1992). This mouse strain harbors the thermolabile SV40 mutation under the control of an IFN-γ inducible H-2K^b promoter. The functional expression of SV40 large T antigen can be turned on by culturing the cells *in vitro* in medium containing IFN-γ at the permissive temperature (33°C). The cells stop proliferating, detach, and die when the temperature is raised to the non-permissive level (39°C) or when the cells are cultured without IFN-γ for three passages. These cells also gradually die at 37°C, although

the onset of cell death is not as rapid as it is at 39°C (Whitehead et al., 1993). These cell lines may be valuable in the study of cell proliferation and differentiation, but their usefulness in the study of mucosal immunology and inflammation is limited.

In this study, we utilized a plasmid vector that contains the origin-deficient SV40 DNA to transfect primary cultured epithelial cells derived from the mouse small intestine and achieved highly efficient expression of large T antigen in epithelial cells. The resulting cell lines have been cloned and characterized. One of the cell clones reported here has been stably immortalized and expresses enterocyte characteristics.

4.2. MATERIALS AND METHODS

4.2.1. Animals

Inbred BALB/c ($H\text{-}2^d$) mice were purchased from Harlan-Sprague Dawley Incorporation (Indianapolis, IN, USA) and bred in our animal facilities in the John P. Robarts Research Institute. 5 to 7 day old mice were used for the primary epithelial cell culture.

4.2.2. Cell culture reagents

DMEM, IMEM, Ham-12 medium, Hanks' balanced salt solution (HBSS), Trypsin-EDTA (0.05% trypsin and 0.53 mM EDTA), and penicillin-streptomycin (1000 U/ml) were purchased from Gibco BRL Laboratories. Collagenase (EC 3.4.24.3, 305 U/mg), insulin (I-5500, 26.2 U/mg), epidermal growth factor (EGF)

(E-4127, 0.1 mg/ml), non-essential amino acids, FCS, PGE_1 (P-7527), triiodothyronine (T3) (T-2627), transferrin (T-2252), sodium selenite (S-1382), and hydrocortisone (H-4001) were purchased from Sigma. Dispase (grade I, Bacillus polymyxa, 6 U/mg) and calf dermal collagen (type I) were purchased from Boehringer Mannheim (Laval, Quebec, Canada). A hormone mixture that contained insulin (500 ug/ml), PGE_1 (125 ng/ml), T3 (3.4 ng/ml), transferrin (500 ug/ml), sodium selenite (173 ng/ml) and hydrocortisone (1.8 ug/ml) was prepared in HBSS, aliquoted, and stored at -70°C.

K1 medium was prepared by mixing DMEM and Ham-12 at 50/50% (vol/vol), and supplemented with the following ingredients: 5% FCS, 1 mM sodium pyruvate, 2 mM glutamine, 1% penicillin-streptomycin, 1% hormone mixture, 1% non-essential amino acid and 10 ng/ml EGF (Wuthrich et al., 1990).

4.2.3. Monoclonal Antibodies

The following monoclonal antibodies were obtained from Cedarlane Laboratories (Hornby, Ontario, Canada): a rat anti-mouse CD54 (clone 3E2), which binds to ICAM-1; a rat anti-mouse $H\text{-}2D^d$ (clone 34-2-12), which binds to MHC class I molecules; a rat anti-mouse $I\text{-}A^d$ (clone AMS-32.1), which binds to MHC class II molecules; and a FITC-conjugated goat anti-rat IgG antibody.

A rat anti-mouse CD3 antibody (clone KT3), which binds to T cells; a rat anti-mouse CD45 antibody (clone 30F 11.1),

which binds to pan leukocyte common antigen found on all lymphocytes, monocytes, and granulocytes; and a mouse anti-SV40 large T antibody (clone PAb 114) was purchased from PharMingen (San Diego, CA, USA). A FITC-conjugated mouse anti-human pan-cytokeratin antibody (clone PCK-26) that cross-react with mouse cytokeratins (cytokeratin 1, 5, 6, 8) and a FITC-conjugated goat anti-mouse IgG antibody were purchased from Sigma. A rat anti-mouse ZO-1 antibody (R40.76) was kindly provided by Dr. R. Shivers (Department of Zoology, University of Western Ontario, London, Ontario, Canada).

4.2.4. Mouse epithelial cell isolation

BALB/c mice (5 to 7 days old) were anaesthetized by hypothermia and the total jejunum and ileum were harvested under sterile conditions. The intestinal tissue was put into a glass petri dish (60 x 15 mm) that contained 2 ml DMEM medium. The intestine was cut into fine segments (0.5 to 1 mm) using a sterile surgical blade and this step was performed on ice. The glass petri dish was used to avoid plastic debris generated by cutting of the intestinal tissue. The tissue segments were transferred into 15 ml centrifugation tubes (polystyrene, Corning, New York, NY, USA) and washed three times in HBSS solution. The tissue segments were resuspended with DMEM containing Dispase (2 U/ml) and collagenase (200 ug/ml) and incubated at 37°C for 30 minutes. After the incubation, the tissue segments were gently pipetted up and down several times using a glass

Pasteur pipette. The mixture that contained tissue debris, epithelial aggregates, and single epithelial cells was allowed to sediment under gravity for a brief period of time to allow the large tissue debris to settle down to the bottom of the tube. The top layer containing intact epithelial aggregates and single epithelial cells was harvested. The supernatant was centrifuged at 300 rpm for 4 minutes to separate the epithelial aggregates from single cells. The same procedure was repeated 4 times until the supernatant became clear. The final cell pellets consisting of purified epithelial aggregates were used for primary culture.

4.2.5. Primary epithelial cell culture

The plastic petri dishes (100 x 20 mm, Corning) were coated with bovine dermal collagen before primary epithelial culture. The stock collagen was diluted with HBSS (without Ca^{2+} and Mg^{2+}) to 200 ug/ml, and then 2 ml was transferred to each dish. The dishes were kept at room temperature for 30 minutes and then the solution was discarded. The purified epithelial aggregates were resuspended in K1 medium and plated in collagen-coated culture dishes. Each dish contained 10 ml K1 medium (approximately 100 to 200 epithelial aggregates). The dishes were cultured in an incubator at 37°C with 5% CO_2 and 100% relative humidity. The cells were fed every 3 days with fresh K1 medium and routinely examined for cell growth under phase contrast microscopy.

When cells reached confluence, the culture medium was discarded and the cells were released from the culture surface by trypsin-EDTA treatment for 10 minutes at room temperature. The cells were washed once in HBSS and resuspended in K1 medium, then split 1:3 for subsequent culture. Under these conditions, the epithelial cells could be maintained for 6 to 7 passages before they become senescent. For cell transfection, care was taken to use the cultures of the second and the third passage, since cells grew vigorously and formed confluent monolayers at this period.

4.2.6. Cell transfection and transfectant isolation

Before cell transfection, the primary cultured cells were fed with .5 ml fresh K1 medium. The origin-deficient SV40 DNA mutant 6-1 in pMK16 plasmid (Gluzman et al., 1980) was prepared from *Escherichia coli* using an alkaline lysis method and further purified using polyethylene glycol precipitation (Ausubel et al., 1989). The primary cultured epithelial cells were transfected using the calcium phosphate coprecipitation method (Sambrook et al., 1989). Briefly, plasmid DNA (10 ug) was precipitated in 2 ml of calcium phosphate solution (125 mM, pH 7.05) at room temperature for 30 minutes. The resulting cloudy solution was gently mixed by pipetting up and down with a Pasteur pipette and then slowly transferred into the culture dish in which primary cultured epithelial cells had formed a confluent monolayer. After the addition of calcium-DNA

coprecipitate, the cultures were incubated in a CO_2 incubator at 37°C overnight. The culture medium with the SV40 DNA was discarded in the next day and the cells were fed with 5 ml fresh K1 medium. Cells were maintained in the culture with weekly changes of fresh K1 medium.

Most of the cells died in the first week after the addition of calcium-DNA coprecipitate since the high concentration of calcium is highly toxic to epithelial cells. Distinct foci of outgrowing cells became apparent four weeks after the transfection. At this stage, cell colonies were carefully marked at the bottom of the culture dish under phase-contrast microscopy. These colonies were detached from the culture surface with an Eppendorf pipette, individually picked up, and transferred to 24-well plates (polystyrene, Corning) that were already coated with collagen. The cell growth was examined daily after the transfer. When cells reached confluence, cells were digested from the culture surface with trypsin-EDTA solution and further propagated in tissue culture dishes (60 x 15 mm, polystyrene, Corning).

4.2.7. Limiting dilution and cell cloning

One cell line obtained with typical epithelial cell morphology were subsequently cloned by a limiting dilution method to ensure single cell origin. This experiment was performed in 96-well, flat-bottom plates (polystyrene, Corning) that were coated with collagen as described above. Epithelial cells harvested from culture dishes were diluted

to 1 x 10^5 cells/ml with K1 medium and 0.1 ml was plated into each well of the plates. When the cells formed a monolayer, the whole plates were irradiated with 3000 rads using a gamma-irradiator to stop the cell proliferation. The irradiation-treated cells were used as the feeder layers. The epithelial cells originating from the same colony were diluted to different concentrations (1, 10, and 100 cells/ml) with K1 medium and 0.1 ml was plated into each well of 96-well plates coated with a monolayer of feeder cells. Plates were incubated at 37°C with 5% CO_2 and 100% relative humidity. Plates were assessed weekly for cell growth and evaluated for proliferating wells at three to four weeks under a phase contrast microscope. The growth frequency of epithelial cells at 1, 10, and 100 cells/ml dilutions was 7.3%, 27%, and 72.9% respectively. Epithelial cells from the lowest dilution were selected, individually transferred to 24-well plates, and further propagated in tissue culture dishes.

4.2.8. Cell proliferation assay

The growth potential of primary cultured and transformed epithelial cells was compared using a cell proliferation assay. Briefly, the primary cultured cells and IEC-4.1 cells were titrated in parallel in K1 medium, plated in collagen-coated 96-well plates (1 x 10^4 cells/well) in triplicate, and incubated in a CO_2 incubator with 100% humidity at 37°C overnight. The cells were pulsed with 1 uCi ^{3}H-TdR (40-60 Ci/mmol, Amersham International, UK) for 18 hours.

The culture medium was discarded and the cells were treated with 100 ul Trypsin-EDTA at room temperature for 10 minutes. The cells were harvested onto fiber glass papers using a automatic cell harvester (Skatron, Sterling, VA, USA). The ^{3}H-TdR uptake by the epithelial cell DNA was quantitated using a scintillation counter (Beckman LS 1701, Glenrothes, UK).

4.2.9. Flow cytometry

The entire cell staining procedure was performed on ice. The epithelial cells (1 x 10^{6}) were incubated with the following primary rat anti-mouse monoclonal antibodies: I-A^{d}, I-D^{d}, ICAM-1, CD3, CD45 for 45 minutes. Heat inactivated rabbit serum (Cedarlane) was used to block the non-specific binding. Cells were washed twice in phosphate buffered saline and further stained with the secondary FITC-conjugated goat anti-rat IgG for an additional 45 minutes. Control preparations were stained with the secondary FITC-conjugated goat anti-rat IgG alone. The fluorescence profiles generated by antibody staining were analyzed using FACScan® (Becton Dickinson Canada, Mississauga, Ontario, Canada) in which a laser beam was used to excite FITC. Files were collected from 2 x 10^{4} cells for each sample, and the measurements of side scatter and forward scatter were stored along with fluorescence profiles. Data were plotted as the number of cells (ordinate) versus the log fluorescence intensity. Channels of background fluorescence intensity observed in the absence

of the primary antibody were mathematically subtracted from each sample before calculating the percent of initial level of surface marker expression. Results were expressed as percentage of positive cells among the total number of cells counted.

4.2.10. Immunofluorescence staining

To determine the expression of cytokeratins, the epithelial cells were cultured to confluence on collagen-coated glass coverslips (20 x 50 mm) and fixed with 2% paraformaldehyde prepared in phosphate buffered saline at room temperature for 2 minutes. The coverslips were washed twice with 50 mM Tris buffer (pH 7.6) and then stained with a FITC-conjugated monoclonal antibody against pan-cytokeratins, diluted 1: 25 in Tris buffer containing 10% heat-inactivated rabbit serum (Cedarlane), for 60 minutes at room temperature, followed by washing of the cells with Tris buffer three times. The murine fibroblast cell line L929 was processed in the same way and used as a control.

For SV40 large T antigen staining, the epithelial cells on glass coverslips were fixed with 2% paraformaldehyde for 2 minutes and the cell membranes were permeabilized with acetone for 45 seconds at room temperature. The cells were stained with a mouse anti-large T antigen antibody for 30 minutes at room temperature and then washed three times in Tris buffer. The cells were further stained with a FITC-conjugated goat anti-mouse IgG for an additional 30 minutes.

The heat inactivated rabbit serum (10%) was used to block the non-specific binding. The epithelial cells stained with the secondary FITC-conjugated goat anti-mouse IgG alone were included as a negative control. The coverslips were washed in Tris buffer, mounted, and examined using a fluorescence microscope (Olympus BH2 system).

For the junctional protein staining, the coverslips with an epithelial monolayer were fixed with 2% paraformaldehyde for 2 minutes and rinsed briefly in phosphate buffered saline. The cell membrane was permeabilized with methanol for 4 minutes and rinsed three times in phosphate buffered saline, then incubated with a rat anti-mouse ZO-1 antibody (R40.76), diluted at 1:250 in phosphate buffered saline, for 60 minutes at room temperature in a humidified chamber. The bovine serum albumin (3%) was added into the phosphate buffered saline to block non-specific binding. The coverslips were washed in phosphate buffered saline after the incubation and further stained with a FITC-conjugated goat anti-rat IgG for 45 minutes. The epithelial cells stained with the secondary FITC-conjugated goat anti-rat IgG alone were used as a negative control. The coverslips were washed three times in phosphate buffered saline, mounted, and examined using a fluorescence microscope (Olympus BH2 system).

4.2.11. Scanning electron microscopy

Epithelial cells were grown on collagen-coated glass coverslips. When cells reached confluence, the coverslips

with an epithelial monolayer were rinsed in phosphate buffered saline for 15 seconds and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffered saline (pH 7.2) at 4°C for 1 hour. The coverslips were washed in 0.1 M phosphate buffered (pH 7.2) twice for 10 minutes and cells were further fixed in 1% aqueous osmium tetroxide in 0.1 M osmium acid (pH 7.2 to 7.4) for 1 hour. The coverslips were then rinsed in distilled water for 10 minutes and dehydrated through a graded series of ethanol (50%, 70%, 90%, 100%, 100%) incubating 10 minutes in each concentration. The coverslips were dried in a critical-point drying apparatus and coated with gold-palladium in a Hummer-VI vacuum coater. The cells were examined using a Hitachi S-100 scanning electron microscope (NSA Hitachi, Mountain View, CA, USA) at an accelerating voltage of 10 kV (Ma et al., 1992).

4.3. RESULTS

4.3.1. Primary epithelial cell culture

In primary epithelial cell culture, the quality and treatment of tissue culture dishes were critical. Tissue culture dishes from several suppliers were screened and it was found the tissue culture treated, polystyrene tissue culture dishes from Corning (catalogue 25020-100) were suitable for optimal epithelial cell growth.

The intact epithelial aggregates attached to the collagen-coated culture surface and spread out to form small islands of outgrowing cells 3 days after primary culture

(Plate 3). Over the next 5 to 7 days, the majority of cells in each island continued to proliferate until confluent monolayers were formed (Plate 4). Under phase-contrast microscopy, cells had a cobblestone-like appearance. When cells reached confluence, the cells were digested from the culture surface using trypsin-EDTA and passaged 1:3 in K1 medium. The cells continued to proliferate after passage and reached confluence within 3 to 4 days. The cells could be maintained in the primary culture for 6 passages, then cells underwent condensation and death.

In most cases, the primary culture resulted in very pure epithelial monolayers. Occasionally, initial epithelial outgrowth was accompanied by fibroblastic contamination which overwhelmed the epithelial colonies if left unchecked. The fibroblastic contamination was eliminated by frequent exposure to trypsin-EDTA treatment. Fibroblasts were more sensitive to this treatment and were rinsed off with HBSS leaving the epithelial cells adherent.

The initial characterization of primary cultured cells demonstrated that almost all cells stained positive for pan-cytokeratins (Plate 5), a cytoplasmic microfilament expressed exclusively by epithelial cells. Phenotypic analysis using FACS showed that the primary cultured cells did not express the lymphoid cell marker such as CD45, but they did express MHC class I (38%) and low levels of MHC class II molecules (20%) (Figure 6).

4.3.2. Cell transfection

One of the three transfection attempts performed was successful. The optimal concentration of plasmid DNA for successful transformation of epithelial cells was 10 ug per 100 x 20 mm culture dish. A higher concentration of plasmid pMK16 DNA did not lead to increased efficiency of cell transfection.

During the first week of transfection, most of the cells died and lifted off the culture surface. In the next few weeks, distinct outgrowing cell foci became evident. Cells were growing rapidly and formed distinct cell colonies with typical cobblestone appearance within the next one or two weeks (Plate 6). Most of the cells had large nuclei and multiple mitotic figures. The position of cell colonies was marked at the bottom of the tissue culture plate under a phase-contrast microscope and carefully transferred into 24-well culture plates that were coated with collagen and the cells were further expanded in the plates. Seven epithelial cell lines were obtained and all stained positive for cytokeratins and expressed typical epithelial morphology.

4.3.3. Cell cloning

Of all 7 cell lines tested, IEC-4 cells were found to constitutively express low levels of MHC class II molecules (Figure 7), and was therefore further cloned using a limiting dilution method. The IEC-4.1 clone was established and further characterized. IEC-4.1 cells exhibited typical

epithelial morphology and uniform in size under phase-contrast microscopy (Plate 7). All the cells had bright nuclear staining of SV40 large T antigens (Plate 8). IEC-4.1 cells still stained positive for this nuclear protein eight months after cell cloning (Plate 9), suggesting that IEC-4.1 had stably integrated the SV40 large T gene into the cell genome.

4.3.4. Growth properties of IEC-4.1 cells

IEC-4.1 cells were cultured and passaged in K1 medium supplemented with 5% FCS, 5 ug/ml insulin, and 10 ng/ml EGF in collagen-coated tissue culture dishes. The cells were adherent to the culture surface 2 hours after plating and underwent rapid cell division. Confluent monolayers were formed within 3 to 4 days after plating (Plate 10). The cell doubling time was approximately 18 hours.

The growth kinetics of IEC-4.1 cells was different from primary cultured epithelial cells. In primary culture, vigorous cell proliferation occurred at 2 to 3 weeks, most of the epithelial cells stopped proliferating and underwent cell death 5 weeks after primary culture. The introduction of SV40 large T gene into epithelial cells resulted in increased growth potential. As shown in Figure 8, five weeks after cell transformation, the growth potential of IEC-4.1 cells continued to increase.

The cell density for optimal proliferation was around 1×10^5 cells/ml. When the cell density was below 1×10^4 cells /ml, cells grew slowly with poor morphology during the

first few days (Plate 11). Coating the culture surface with collagen was not necessary for IEC-4.1 cells since IEC-4.1 cells grew well without the collagen coating. The morphology was almost identical to those growing in collagen coated dishes. IEC-4.1 cells also grew vigorously in K1 medium without EGF (Plate 12), but cells always had better morphology with the addition of EGF. IEC-4.1 cells have been growing in the dish for more than one year.

4.3.5. Phenotype of IEC-4.1 cells

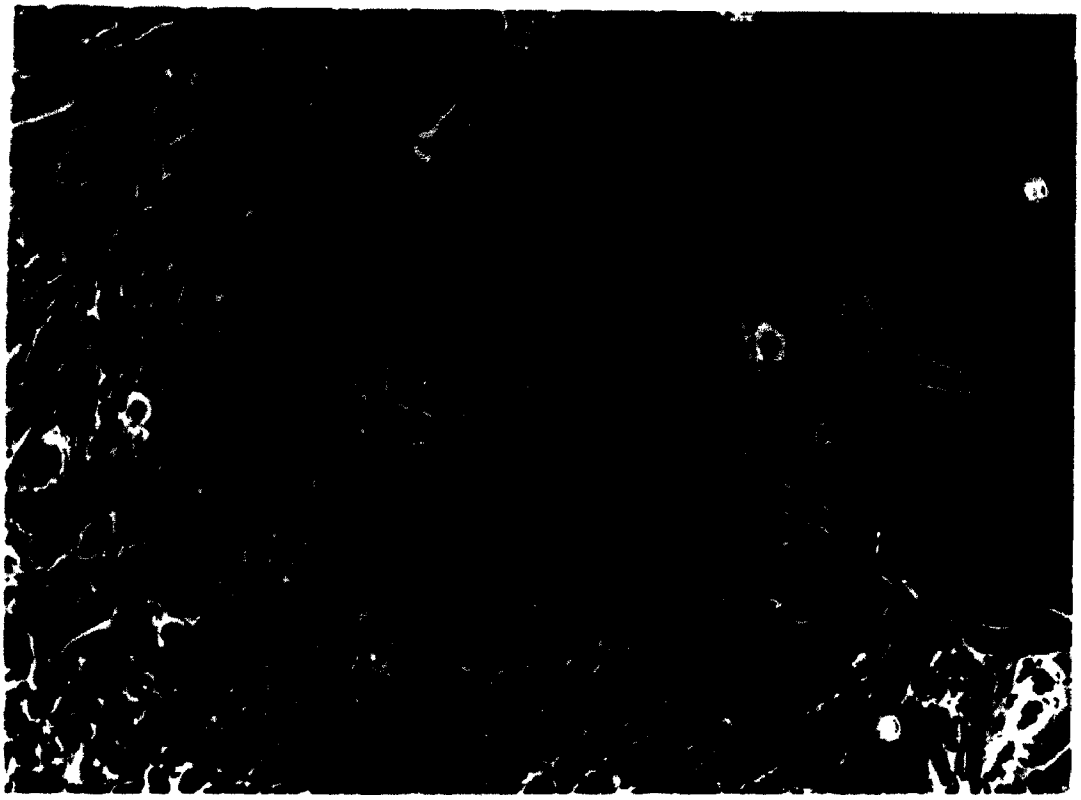
FACS analysis showed that IEC-4.1 cells did not express the lymphoid cell marker such as CD45, but they did express MHC class I (98%) and low levels of MHC class II molecules (20%). IEC-4.1 cells also expressed adhesion molecules ICAM-1 (14%) under normal culture conditions (Figure 9).

Immunofluorescence staining demonstrated that IEC-4.1 cells stained positive for pan-cytokeratins (Plate 13). When IEC-4.1 cells grew at sub-confluence, low intensity of ZO-1, a junctional protein that is associated with the formation of tight junctions between epithelial cells, was present at the borders of the cells. Over the next few days, the intensity and distribution of ZO-1 fluorescence became progressively stronger. On the confluent monolayers, ZO-1 was distributed along the periphery of IEC-4.1 cells, suggesting the formation of tight junctions at confluence (Plate 14).

4.3.6. Ultra-structural characteristics of IEC-4.1

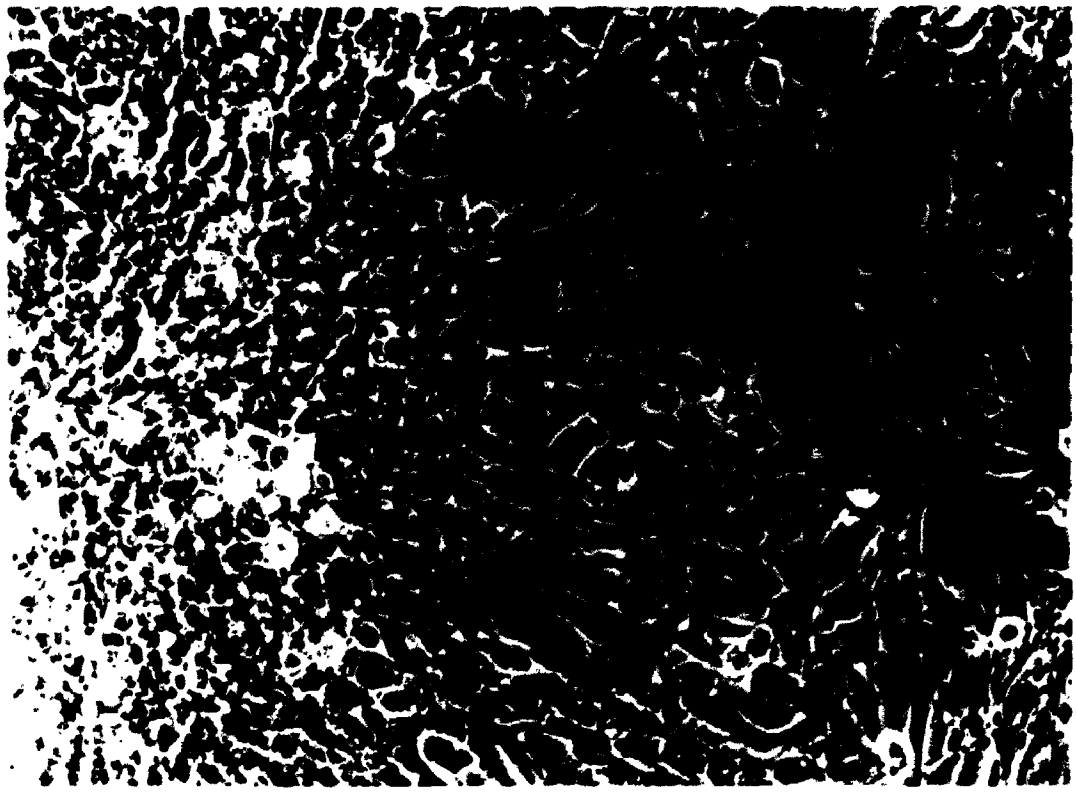
IEC-4.1 cells were examined using scanning electron microscopy. At the sub-confluent stage, cells were well spread and flat, the cytoplasmic extension of adjacent cells made focal or uniform tight contacts with one another. Fusion structures between adjacent cells were observed, but wide intercellular spaces were often present. The microvilli were present on the cell surface but at low density at sub-confluence (Plate 15). The morphologic characteristics changed at confluence. The cells exhibited a higher density of microvilli on the cell surface and formed tight contacts with adjacent cells (Plate 16).

Plate 3. Epithelial cells in primary culture



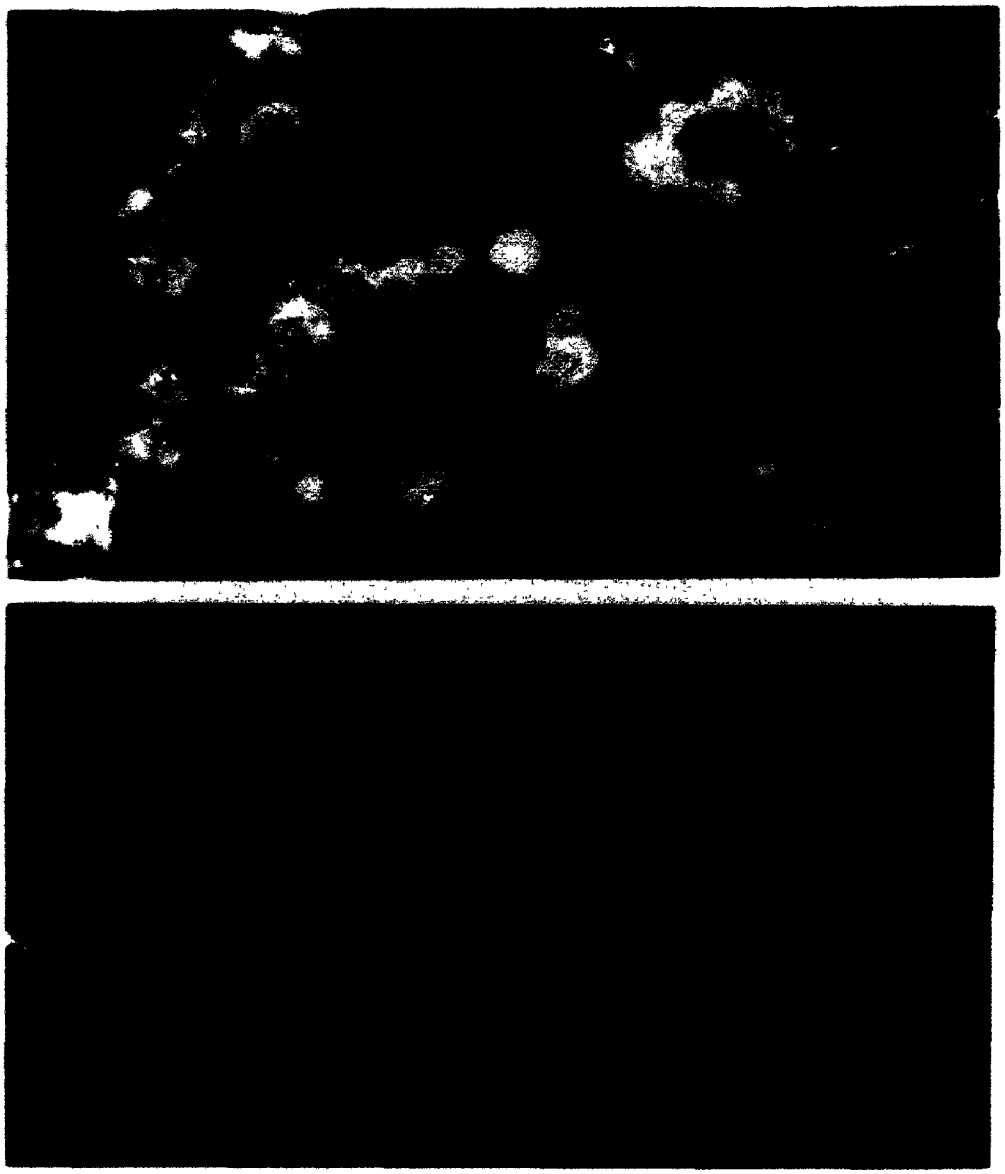
The morphology of one outgrowing epithelial cell island 3 days after primary culture. 150 X.

Plate 4. Monolayer of epithelial cells in primary culture



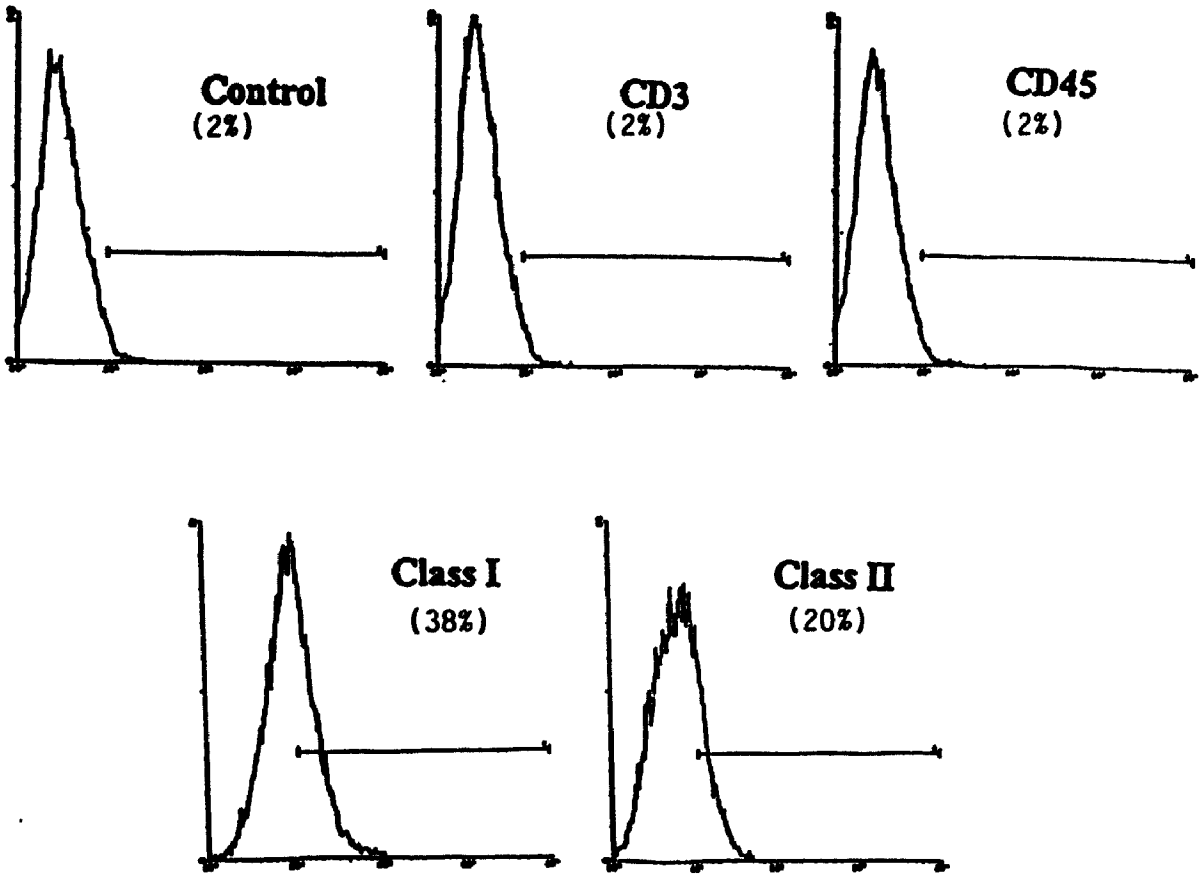
The morphology of small intestine-derived epithelial cells 5 days after primary culture. 150 X.

Plate 5. Cytokeratin staining of primary cultured intestinal epithelial cells



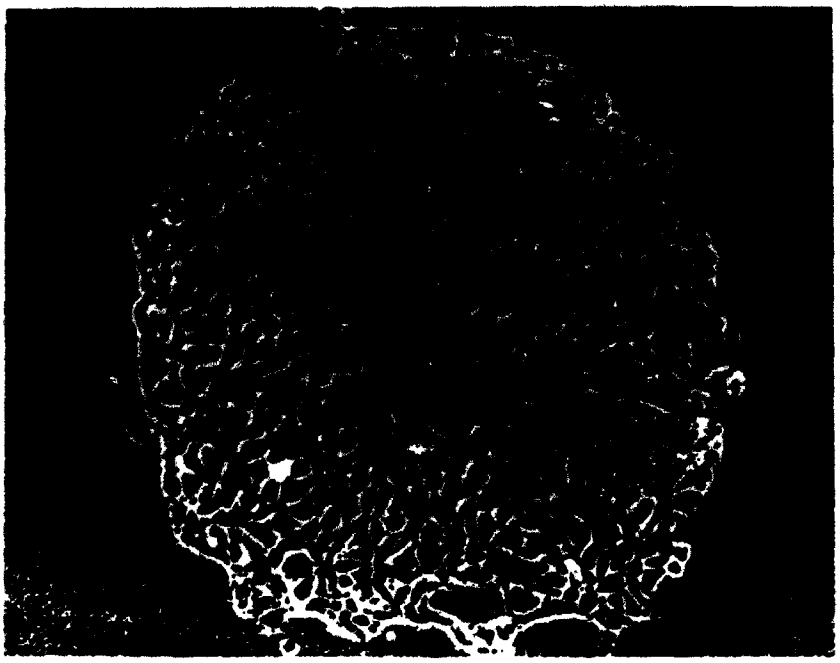
A: Primary cultured epithelial cells stained with the FITC-conjugated anti-cytokeratin antibody. B: A fibroblastic cell line L929 stained with the FITC-conjugated anti-cytokeratin antibody as a negative control. 135 X.

Figure 6. FACS analysis of primary cultured intestinal epithelial cells



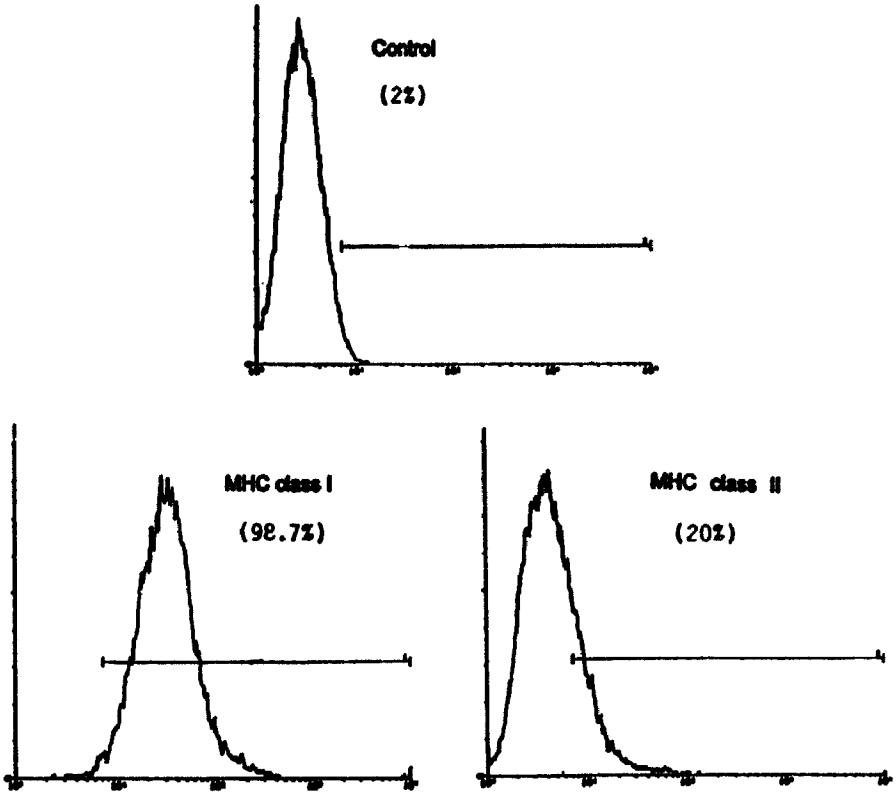
Cells (10^6) were stained with the rat anti-mouse monoclonal antibodies and further stained with the FITC-conjugated goat anti-rat IgG after washing in phosphate buffered saline. Control preparations were stained with the FITC-conjugated goat anti-rat IgG alone. The fluorescence profiles generated by the staining were analyzed using FACScan. Files were collected from 2 x 10^4 cells for each sample, and data were plotted as the number of cells (Y axis) versus the log fluorescence intensity (X axis). Results are expressed as percentage of positive cells among the total number of cells counted.

Plate 6. Morphology of epithelial cell colonies after transfection



The morphology of one typical epithelial cell colony under phase contrast microscopy 3 weeks after transfection. 150 X.

Figure 7. Expression of MHC class I, MHC class II on IEC-4 epithelial cell line

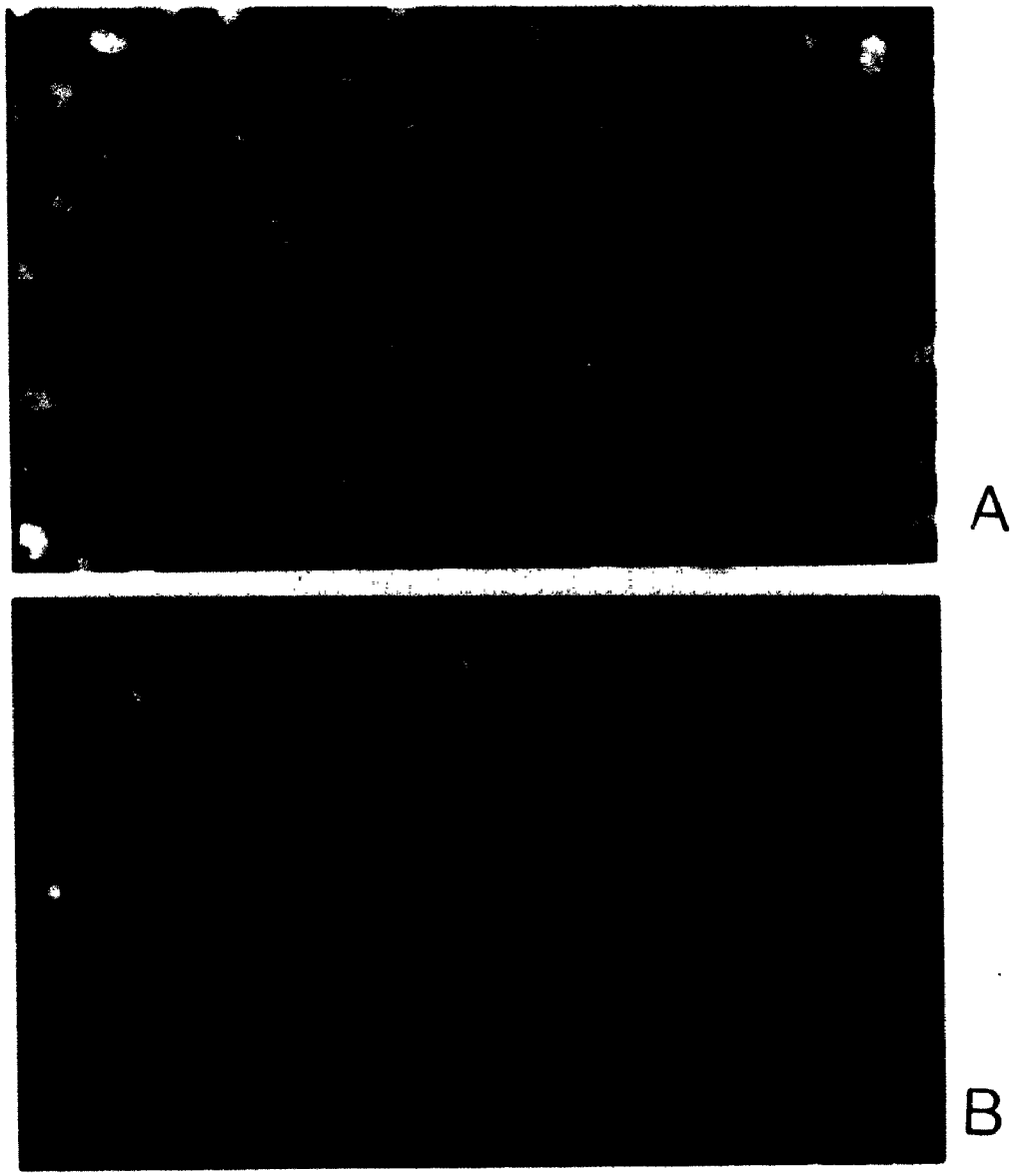


Cells (10^6) were stained with the rat anti-mouse MHC class I or class II antibody and further stained with the FITC-conjugated goat anti-rat IgG after washing in phosphate buffered saline. Control preparations were stained with the FITC-conjugated goat anti-rat IgG alone. The fluorescence profiles generated by the staining were analyzed using FACScan. Files were collected from 2 x 10^4 cells for each sample, and data were plotted as the number of cells (Y axis) versus the log fluorescence intensity (X axis). Results are expressed as percentage of positive cells among the total number of cells counted.

Plate 7. Morphology of IEC-4.1 clone under phase contrast microscopy

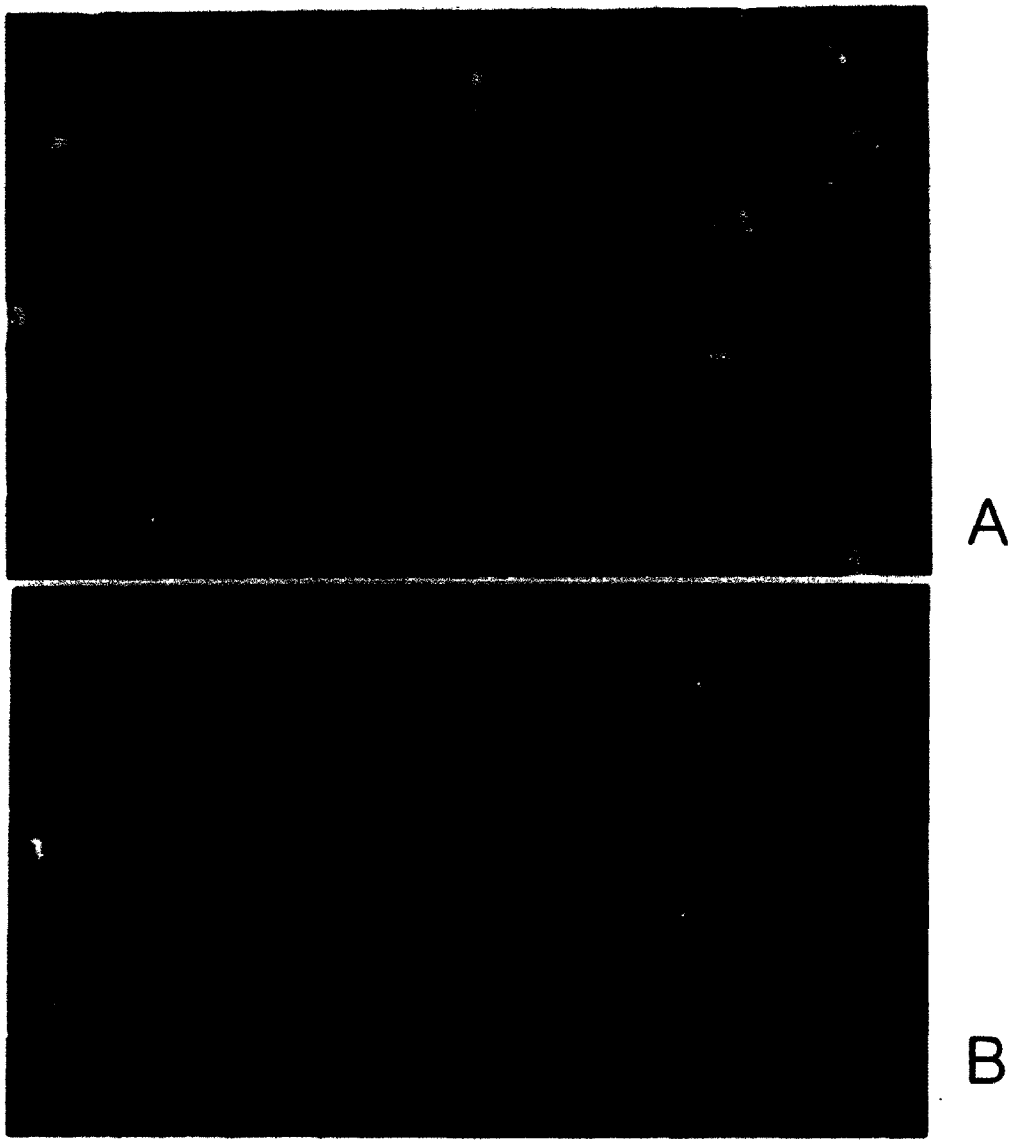
The morphology of IEC-4.1 cells two days after passage. 150 X.

Plate 8. SV40 large T antigen staining of IEC-4.1 cells



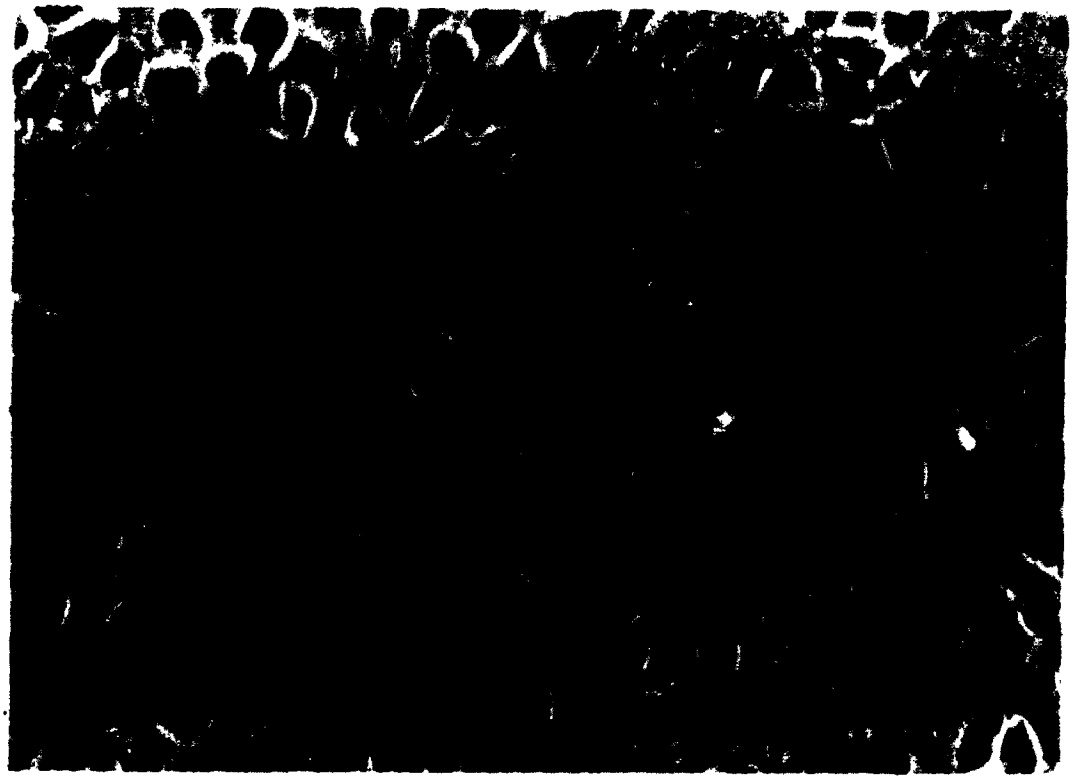
A: SV40 large T antigen staining two weeks after cell cloning showing the bright nuclear staining of SV40 large T antigen. IEC-4.1 cells were stained with a mouse anti-large T antibody and further stained with FITC-conjugated goat anti-mouse IgG after washing. B: Cells stained with the FITC-conjugated goat anti-mouse IgG alone as a negative control. 135 X.

Plate 9. Stable expression of SV40 large T antigen in IEC-4.1 cells



A: SV40 large T antigen staining in IEC-4.1 cells eight months after cell cloning. B: Cells stained with the FITC-conjugated secondary antibody alone as a negative control. 135 X.

Plate 10. Morphology of IEC-4.1 cells in confluent monolayer

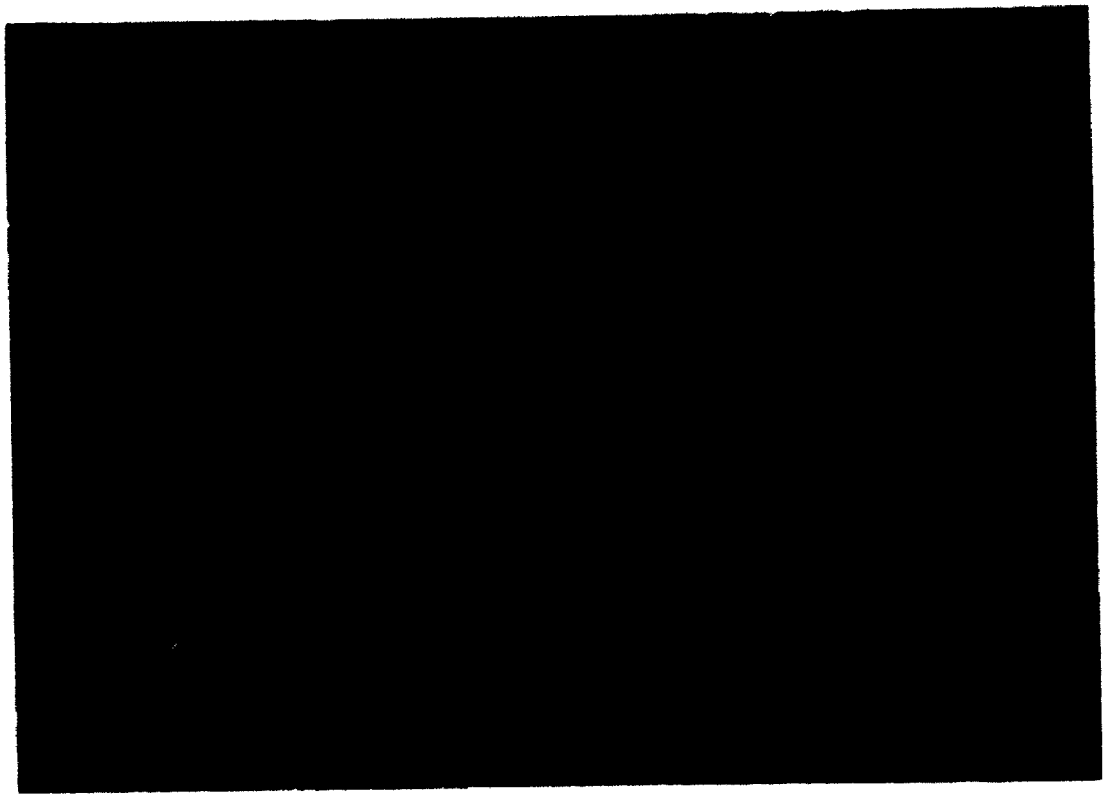


Morphology of IEC-4.1 cells in confluent monolayer under phase contrast microscopy. 150 X

Plate 11. Morphology of IEC-4.1 cells passaged with low cell density

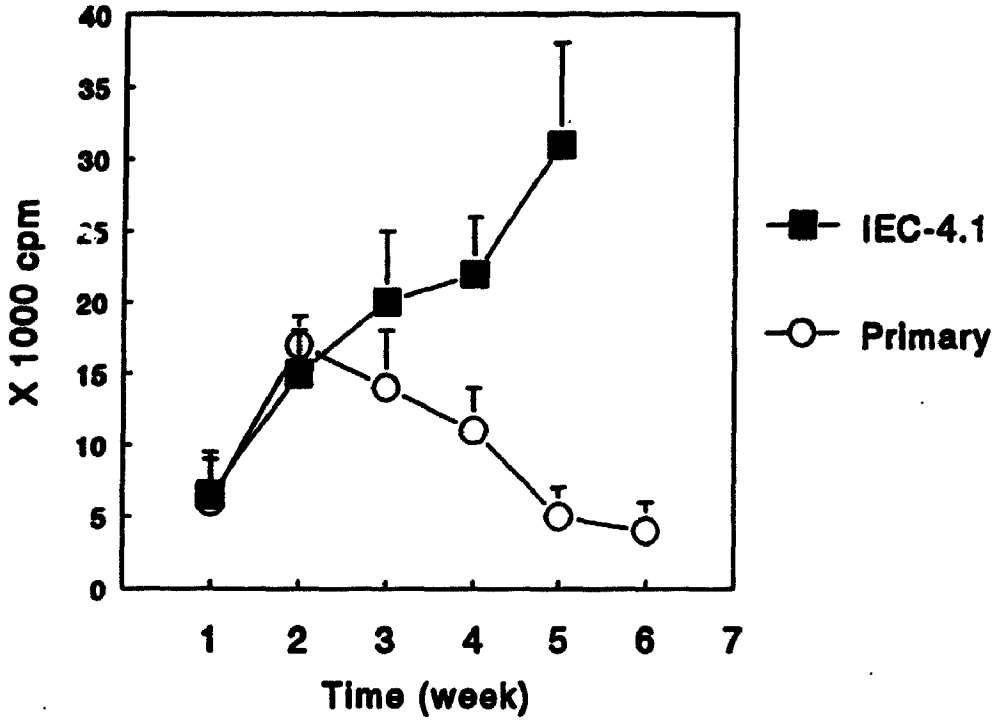
Morphology of IEC-4.1 cells 3 days after passage with the initial cell density below 1 X 10^4 cells/ml. 150 X.

Plate 12. Morphology of IEC-4.1 cells without EGF



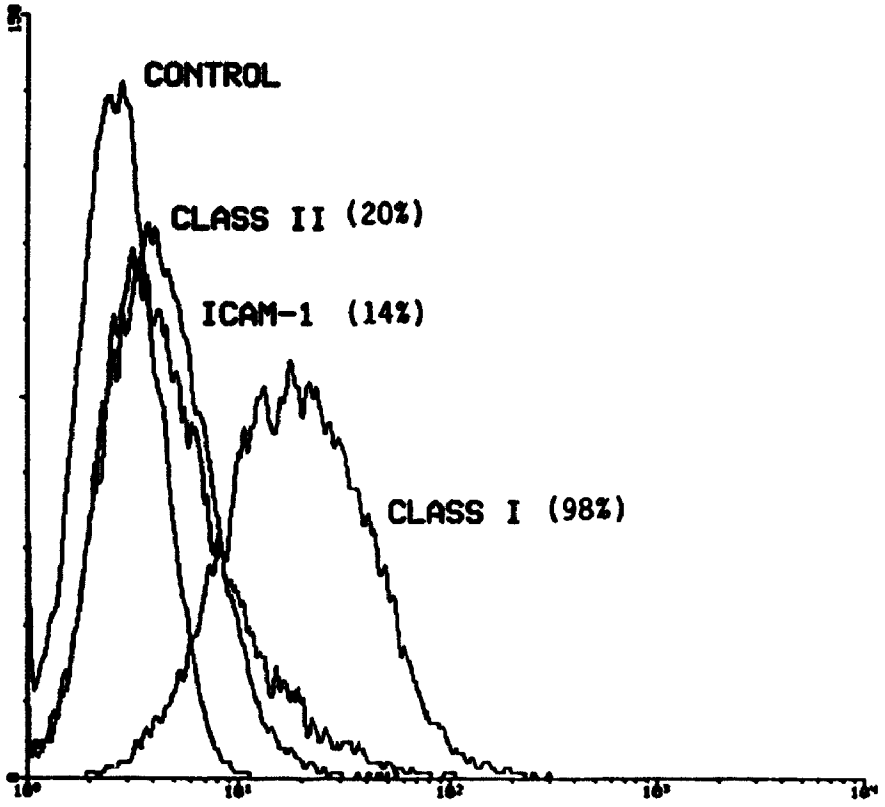
Morphology of IEC-4.1 cells cultured in K1 medium without EGF supplementation. 150 X

Figure 8. Comparison of growth kinetics between primary cultured and transformed epithelial cells



The growth potential of primary cultured and transformed epithelial cells (IEC-4.1) was compared in a cell proliferation assay. The Y axis is the cpm counts and the X axis is the time points examined. The data are presented as mean cpm $\pm$ SD of triplicate assays.

Figure 9. FACS analysis of IEC-4.1 cells

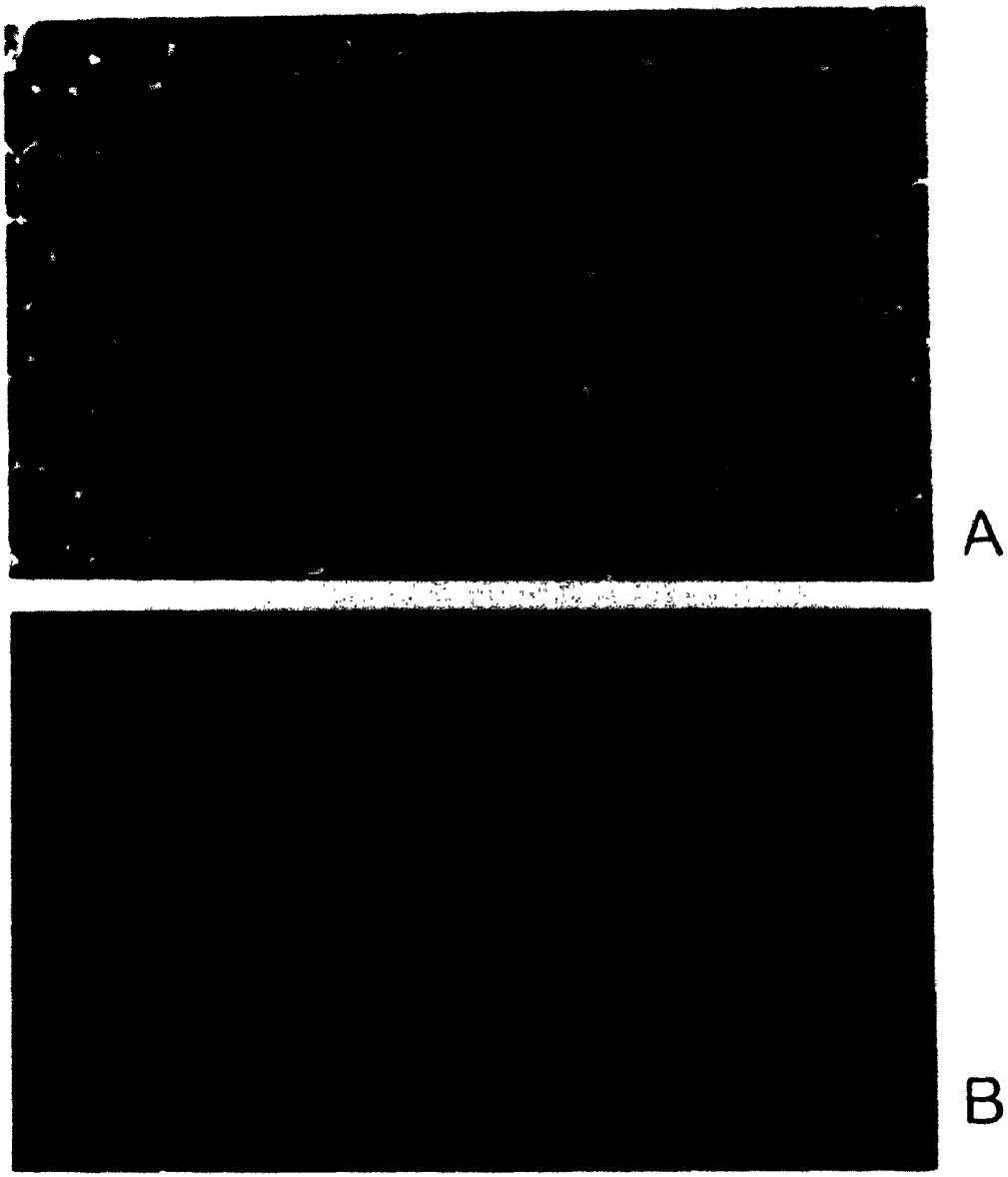


IEC-4.1 cells (10^6) were stained with the rat anti-mouse MHC class I, class II or ICAM-1 antibody and further stained with the FITC-conjugated goat anti-rat IgG after washing in phosphate buffered saline. Control preparations were stained with the FITC-conjugated goat anti-rat IgG alone. The fluorescence profiles generated by the staining were analyzed using FACScan. Files were collected from 2 x 10^4 cells for each sample, and data were plotted as the number of cells (Y axis) versus the log fluorescence intensity (X axis). Results are expressed as percentage of positive cells among the total number of cells counted.

Plate 13. Cytokeratin staining of IEC-4.1 cells

A: IEC-4.1 cells stained with the FITC-conjugated anti-cytokeratin antibody showing the typical pattern of cytokeratin staining (135 X). B: A fibroblastic cell line L929 stained with the FITC-conjugated anti-cytokeratin antibody as a control (62.5 X).

Plate 14. Expression of ZO-1 protein by IEC-4.1 cells

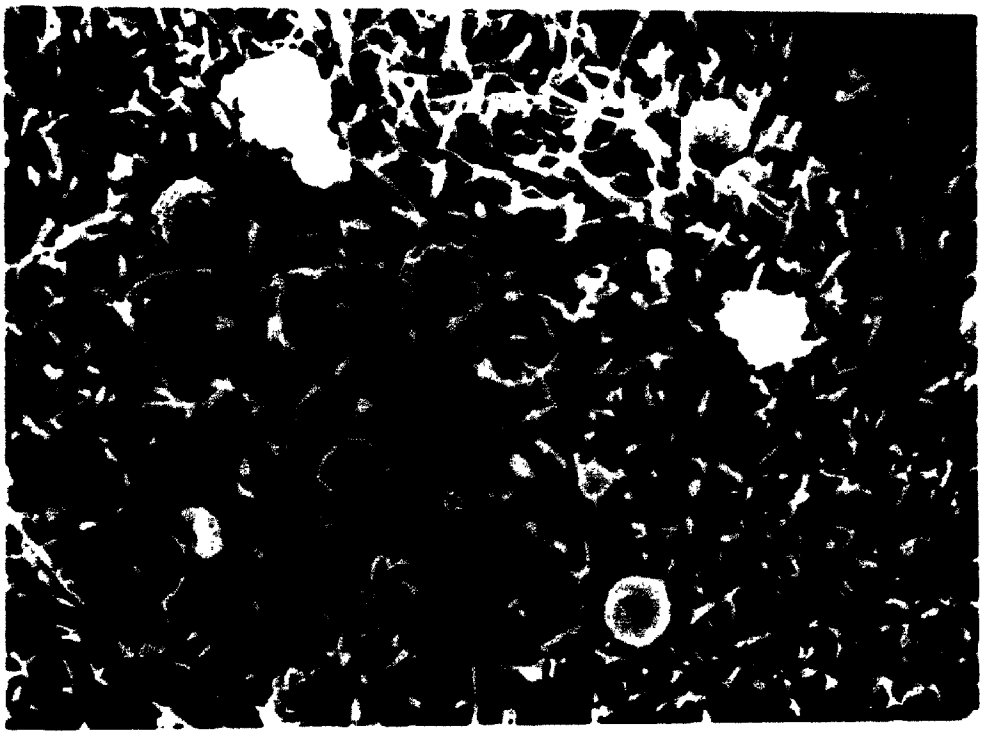


A: IEC-4.1 cells were stained with a rat anti-mouse ZO-1 antibody at confluence (5 days after passage) and further stained with FITC-conjugated goat anti-rat IgG after washing. ZO-1 was distributed along the cell periphery. B: IEC-4.1 cells stained with the FITC-conjugated goat anti-rat IgG alone as a negative control. 135 X.

Plate 15. Presence of microvilli on IEC-4.1 cells

Scanning electron microscopy showing the expression of microvilli on the surface of IEC-4.1 cells at sub-confluence. The arrow shows the intercellular space at this stage. 8,000 X.

Plate 16. Microvilli on IEC-4.1 cells at confluence



Scanning electron microscopy showing the increased density of microvilli on the surface of IEC-4.1 cells at confluence. 6,000 X.

4.4. DISCUSSION

The intestinal epithelium has attracted considerable research interest. It harbors a unique population of T cells expressing γ/δ TCR and has been shown to be a primary lymphoid organ with the capacity to positively and negatively select developing T cells (Sollid et al., 1988; Croitoru et al., 1992). The epithelial cells are also in a state of rapid proliferation and differentiation along the crypt-villus axis in a highly regulated but poorly understood fashion. Recently, the epithelial cells have been implicated as active participants in the pathogenesis of autoimmune enterocolitis (O'Farrelly et al., 1992). Further studies in this area will help to understand the unique microenvironment, the immunology, and the physiology of intestinal epithelium.

The lack of successful methods to culture intestinal epithelial cells *in vitro* hampers the study of this important cell type. Nearly a dozen transfection techniques have been devised to try to transform epithelial cells of the small intestine, all of which involve the use of calcium phosphate or dextran (or its analogs) as a carrier to deliver DNA into the cells. In some methods, osmotic shock or treatment with lysosomal inhibitors is used to enhance the transfection frequencies. Recently, an electroporation method involving the use of high voltage electric pulses to create pores in the cell membrane has been used to deliver DNA into the epithelial cells. Although these methods are

used with varying degrees of success, the transfection frequencies are relatively low (ranging from 0.001 to 1% depending on the method used) and stable cell transformation is rare (Chen et al., 1987). Cell transfection relies on integration of the exogenous DNA into the genome of target cells and this is dependent on the active proliferation of the target cells. Almost all intestinal epithelial cells die within the first 24 hours in conventional primary epithelial cultures. Therefore, there is very little chance for the exogenous DNA integration to occur.

In this study, the epithelial isolation and culture conditions were optimized and vigorous cell growth was achieved in the primary epithelial culture. The isolation of epithelial aggregates is critical for subsequent cell growth. It appears that the initial epithelial growth in the primary culture requires intimate cellular contact. The isolation of intact epithelial aggregates is highly dependent on the concentration of enzymes used and the duration of digestion. Higher concentrations of enzymes (Dispase >2 U/ml and collagenase >200 ug/ml) and longer digestion time (>30 minutes) result in single cell suspensions that uniformly die within 24 hours of culture.

The collagen coating of culture dishes is also important in primary epithelial cell culture because collagen is one of the major components of the basement membrane and has an intimate relationship with epithelial cell growth. The initial cell growth requires attachment to the culture

surface and collagen can effectively promote this process. The hormone mixture in K1 medium, which contains insulin, PGE_1, T3, sodium selenite, and hydrocortisone, is specially formulated for epithelial cells, and this may be an another reason for the optimal epithelial cell growth observed in this study.

Stable transfection of enterocytes with plasmid DNA containing the SV40 large T gene transcriptional unit has been demonstrated. The SV40 DNA mutant 6-1 used in this study is unable to replicate by itself because the first 6 base pairs of DNA at the origin site (BglI) were deleted (Gluzman et al., 1980). Thus, the SV40 DNA is capable of transforming cells *in vitro* without causing cytotoxic effects. Several factors appear to influence the efficiency of cell transformation, the most important ones being the formation of a polarized epithelial monolayer and the state of vigorous proliferation of epithelial cells in the primary culture. Another factor is the purity and concentration of the plasmid DNA. The plasmid DNA used in the first two transformations was a crude preparation from pMK16 plasmid, which might be one of the reasons for the inefficiency of cell transformation.

The stable cell transformation depends on the constitutive expression of large T antigen, an oncoprotein that can eliminate proliferation controls operating in the G1 phase of the cell cycle. The large T antigen is known to complex with retinoblastoma and p53 tumor suppressor genes,

and inactivates their function (Dyson et al., 1990). The expression of SV40 large T antigen has a profound effect on the epithelial cell growth. The transfected epithelial cells can grow even without growth factors and extracellular matrix proteins. The cell lines obtained have stably integrated the SV40 viral DNA and constitutively express the large T antigen. These cells have been maintained in culture for more than one year. Although all the cell lines are stably immortalized by SV40, they do not display identical features. Variables that account for such differences include the number of copies of the SV40 large T antigen gene incorporated, the region where the gene is inserted into the genome, and the stage of cell differentiation where the cell transformation occurred.

The integration of the SV40 large T gene into epithelial cells drives cells into S phase, but this process has no apparent effect on the cell differentiation program, since the cellular and regional patterns of enterocytic differentiation appear unaffected. It has been shown that the differentiation process of epithelial cells in transgenic mice that express the SV40 large T antigen is similar compared to their normal littermates and the epithelial cell proliferation is not associated with the development of gut neoplasm (Hauft et al., 1992). The expression of large T antigen in cardiac muscle cells enables proliferation of cells with maintenance of the differentiated features such as beating in response to

beta-adrenergic agents and expression of the myosin light chain (Sen et al., 1988).

IEC-4.1 cells are derived from mice with a well characterized genetic background, and this cell clone may offer an unique model to study the immunology of epithelial cells. This cell clone may also be valuable to study the regulatory mechanism involved in epithelial cell proliferation and differentiation. The possibility of losing differentiated cell features may occur with long-term culture although this has not been observed with IEC-4.1 cells. It should be added that long-term *in vitro* culture of these cells may alter their function and phenotype, and thus their response to exogenously applied agents may not be the precise reflection of the *in vivo* situation. Nevertheless, IEC-4.1 cells provide a homogenous epithelial population to address some of the fundamental questions in epithelial damage during graft rejection. We used IEC-4.1 cells to investigate the enterocytotoxic effect of TNF-α and the molecular basis of macrophage-epithelial interactions as described in the following chapter.

CHAPTER 5. CHARACTERIZATION OF INTESTINAL EPITHELIAL CELLS IN RESPONSE TO ENDOTOXIN AND CYTOKINES

5.1. INTRODUCTION

Acute intestinal rejection is associated with elevated levels of endotoxin, IL-6, TNF-α, and massive infiltration of macrophages in the intestinal epithelium, followed by progressive epithelial damage characterized by villus atrophy, crypt hypertrophy, and crypt cell necrosis. Recent studies suggest that cytokines may play a critical role in epithelial damage. In explants of human fetal intestine, the activation of mucosal T cells with mitogen or anti-CD3 antibody induced a profound crypt cell hyperplasia and progressive villus atrophy. These changes were not seen when fetal gut aged 14 weeks (in the total absence of T cells) was stimulated with mitogen (MacDonald, 1992a). The exact mechanisms whereby cytokines damage the gut epithelium remain highly speculative. Activated T cells produce IL-2 and IFN-γ, but neither of these cytokines is enteropathogenic when added to the fetal intestinal explant and the enteropathy induced by polyclonal T cell activation in the intestinal explant can not be prevented by antibodies to these cytokines (MacDonald, 1992a). It appears that the enteropathy involves the participation of other factors.

The precise role of epithelial cells in mucosal inflammation, particularly in regulating leukocyte

trafficking during intestinal rejection, remains to be defined. Studies of leukocyte and endothelial interactions indicate that adhesion molecules participate in leukocyte migration from blood vessels to the site of inflammation. Leukocyte adhesion and migration is a multiple step process and involves several types of adhesion molecules. ICAM-1 is constitutively expressed on endothelial cells and serves as the ligand for LFA-1 on leukocytes. ICAM-1 expression is upregulated by TNF-α, IL-1, and IFN-γ (Springer, 1990). VCAM-1 belongs to the immunoglobulin gene superfamily and its expression is induced on postcapillary venules of inflamed tissue (Briscoe et al., 1992) and on capillaries induced by TNF-α, IL-1, and IL-4 (Thornhill et al., 1991). Its ligand is VLA-4 that is expressed on many cell types, including mononuclear leukocytes and macrophages (Springer, 1990). Endothelial cells also respond rapidly to inflammatory stimuli by mobilizing P-selectin and E-selectin, which interact with neutrophils and promote cell adhesion. Many of these molecules may be expressed by epithelial cells, but little is known regarding enterocytes.

Although structural studies of leukocyte transmigration suggest that adhesive events may be required in leukocyte-epithelial cell interaction, the molecular basis of leukocyte-epithelial interactions has, in general, not been well characterized. This study was undertaken to examine the effect of endotoxin and TNF-α on epithelial cells using IEC-4.1 cells as an *in vitro* system and to further examine

the molecular basis of macrophage adhesion to epithelial monolayers.

5.2. MATERIALS AND METHODS

5.2.1. Reagents

Human recombinant IL-6 was purchased from Bioproducts for Science Inc, (Indianapolis, IN, USA). Human recombinant TNF-α (0.5 mg/ml) was purchased from Genentech, Inc. (San Francisco, CA, USA). FITC-conjugated goat anti-rat IgG were purchased from Cedarlane (Hornby, Ontario, Canada).

The rat anti-mouse monoclonal antibodies against ICAM-1 (YN1/1.7.4, IgG2b), VCAM-1 (M/K-2.7, IgG1), LFA-1 (M17/5.2, IgG2b), VLA-4 (R1-2, IgG2b), CD4 (GK1.5, IgG2b), and the specific mouse cDNA probes for IL-1α, β (Gray et al.,1986), IL-6 (Chiu et al., 1988), TNF-α (Fransen et al., 1985), TGF-β (ATCC 59955), ICAM-1 (Siu et al., 1989), VCAM-1, β-actin (Alonso et al., 1986) were kindly provided by Dr. Anthony Jevnikar (Department of Nephrology, University Hospital, University of Western Ontario, London, Ontario, Canada).

Actinomycin D (A-1410), diethypyrocarbonate (DEPC, D-5758), ethidium bromide (E-8751), guanidinium isothiocyanate (G-7028), LPS (*Escherichia Coli*, 0111:B4), thioglycollate (T-9032) were obtained from Sigma. Agarose (electrophoresis grade), bovine serum albumin, oligonucleotide buffer, Klenow DNA polymerase (6500 u/ml) were purchased from Gibco BRL.

^{32}P-dCTP (10 uCi/ul) was from New England Nuclear/Dupont, Boston, MA, USA. Sephadex G-50 columns were from Paramecia, Wilwaukee, WI, USA. X-ray films (X-OMAT) were from Kodak, Rochester, NY, USA. Hybond N+ nylon membranes were from Amersham International, UK.

5.2.2. Culture of IEC-4.1 cells

IEC-4.1 cells were cultured in K1 medium in collagen-coated tissue culture dishes and passaged by trypsin-EDTA digestion. For RNA extraction, cells were stimulated with LPS (12 ug/ml) or TNF-α (2.5 ng/ml) for 1, 2, 4, 8, 12, 24, or 48 hours. The cells were then harvested and washed once in HBSS by centrifugation at 2000 rpm for 5 minutes. The final cell pellets (approximately 6 x 10^6 cells) were used for RNA extraction. For the cell adhesion assay, IEC-4.1 cells were grown in collagen-coated 96-well, flat bottom tissue culture plates. In some experiments, the epithelial monolayers were treated with LPS (12 ug/ml) or TNF-α (2.5 ng/ml) for 4, 8, 12, or 24 hours before the adhesion assays.

5.2.3. Enterotoxicity assay

The potential effect of endotoxin and TNF-α on intestinal epithelial cells was examined *in vitro* using IEC-4.1 cells. IEC-4.1 cells were cultured in collagen-coated, 96-well, flat-bottom tissue culture plates (1 x 10^4 cells/well) at 37°C for 24 hours. Before the addition of endotoxin or cytokine, the cells were briefly treated with actinomycin D, a transcriptional inhibitor, at

a final concentration of 1 ug/ml for 2 hours. The plates were washed twice in HBSS, and then the serial diluted cytokine or endotoxin were added to each well (100 ul/well) in triplicate. The plates were further incubated for 18 hours. The cell death was determined using the colorimetric assay (Mosmann et al., 1989) as described on page 49. Cells cultured with medium alone were included as a negative control and cells cultured in the presence of 200 ng/ml TNF-α were used as a positive control.

5.2.4. Total cellular RNA extraction

The total cellular RNA was extracted by a guanidinium isothiocyanate-phenol-chloroform extraction method (Feinberg et al., 1983). IEC-4.1 cells were harvested from tissue culture dishes by trypsin-EDTA digestion and washed once in HBSS by centrifugation at 2000 rpm for 5 minutes. The supernatant was discarded and the final cell pellets were lysed with 2.4 ml denaturing solution consisting of 4 M guanidinium isothiocyanate, 0.75 M sodium citrate (pH 7.0), 10% sarkosyl, and 0.1 M 2-mercaptoethnol. The cell lysate was homogenized briefly using a cordless homogenizer (Dremel, Racine, WI, USA) and the following solutions were added sequentially: 240 ul 2 M sodium acetate (pH 4.0); 2.4 ml phenol saturated in water; and 480 ul chloroform-isoamyl alcohol (49:1). The cell lysate was mixed well after each addition and all these steps were performed on ice. The cell lysate was left on ice for 5 to 10 minutes and then centrifuged at 5000 rpm for 15 minutes. The top aqueous

layer was carefully harvested and the total cellular RNA was extracted with chloroform-isoamyl alcohol. RNA was precipitated twice in isopropanol overnight at -20°C. The final RNA pellet was washed twice in 75% ethanol prepared using DEPC-treated water through centrifugation at 15,000 rpm for 15 minutes at 4°C. The final cellular RNA was dissolved in 100 ul DEPC-treated water and stored at -70°C. The control cellular RNA was extracted from the spleen of BALB/c mice 6 hours after LPS (10 ug/mouse) challenge intraperitoneally.

5.2.5. Quantitation and analysis of cellular RNA

Two ul RNA was diluted with 498 ul distilled water (the final dilution was 1:250) in fresh Eppendorf tubes (DiaMed Lab Supplies). RNA was quantitated using a spectrophotometer (Spectronic 1001 plus, Milton Roy company, Rochester, NY, USA) based on the absorbance at 260 nM and 280 nM. The amount of RNA extracted in each sample in ug was calculated using the following formula: OD_{260} x 40 (constant) x dilution x total volume.

The structural integrity of RNA was assessed using agarose gel electrophoresis. Agarose gel (1%) was prepared in Tris-acetate-EDTA (TAE) buffer and boiled until the agarose was completely melted. Ethidium bromide (0.1 ul/ml gel) was added into the agarose gel before pouring into a gel box. The RNA samples were prepared by diluting 1 ul RNA with 3.3 ul DEPC-treated water in Eppendorf tubes (DiaMed Lab Supplies). Before RNA samples were loaded into each well

of the agarose gel, 0.7 ul loading buffer containing 0.25% bromophenol blue, 50% glycerol, 10 mM sodium phosphate, and 0.25% xylene cyanol was added into each tube. The agarose gel was run at 60 volts for one hour in TAE buffer and the gel was examined under ultraviolet light. The presence of 18S and 28S ribosomal bands indicated the integrity of the RNA (Plate 17).

5.2.6. Northern blot analysis

Total cellular RNA (20 ug) was lyophilized in a SpeedVac (Savant Instruments, Farmingdale, NY, USA) and dissolved in a solution containing 0.1 M sodium phosphate (pH 7.0), dimethyl sulfoxide, and 6.0 M deionized glyoxal. RNA samples were denatured at 55°C for 60 minutes and quickly immersed in ice for 10 minutes, then electrophoresed through a 1% agarose gel at 65 volts for 3 hours and 45 minutes in sodium phosphate buffer (10 mM, pH 7.0). RNA samples were transferred from the gel onto nylon membranes using a vacuum blotting system (Vacugene, Uppsala, Sweden) at 40 millibar pressure with 10 mM sodium hydroxide for 15 minutes and 100 mM sodium phosphate (pH 7.0) for 75 minutes. After the transfer, nylon membranes were briefly rinsed in 5 x SSC solution and DEPC-treated water, and finally baked at 80°C for 2 hours to allow permanent binding of RNA to the membranes. The nylon membranes were stored at -80°C before hybridization.

5.2.7. cDNA probes and hybridization

Specific mouse cDNA fragments for IL-1α, IL-1β, IL-6, TNF-α, TGF-β, ICAM-1 and VCAM-1 were labeled by random priming technique with ^{32}P-dCTP (Noronha et al., 1993). Briefly, 20 to 25 ug of cDNA fragment was boiled in 16 ul distilled water in 1.5 ml Eppendorf tubes (DiaMed Lab Supplies) for 10 minutes, quickly immersed in ice for another 10 minutes, and then mixed with the following reagents: 5 ul OLB buffer, 1 ul bovine serum albumin solution (10 ng/ml), 0.5 ul Klenow DNA polymerase (6500 u/ml), and 2.5 ul ^{32}P-dCTP (10 uCi/ul). The random priming reaction was maintained at room temperature for 3 hours and then stopped with 100 ul stop buffer (4 M sodium chloride, 1 M Tris-HCI, 0.2 M EDTA, 10% SDS, and 1 mM dCTP). The radiolabelled cDNA probe was further purified through a Sephadex G-50 column. The specific activity of cDNA probes purified from the column was about 10^8 cpm/ug cDNA fragments.

Hybridization was performed at 65°C in a solution containing 1 mM EDTA, 0.5 M NaH_2PO_4 (pH 7.2), and 7% SDS for a minimum of 12 hours in a rotating hybridization oven (Robbins Scientific, Sunnyvale, CA, USA). Following hybridization, the membranes were washed in a solution containing 1 mM EDTA, 40 mM NaH_2PO_4 (pH 7.2), and 5% SDS for two 30 minute intervals at 65°C and washed in an identical solution containing 1% SDS for two additional 30 minutes intervals at 65°C. The membranes were mounted on dry filter

paper and exposed to Kodak X-ray film with intensifying screens at -70°C for 2 to 7 days.

5.2.8. Macrophage preparation

BALB/c mice were treated with 1 ml thioglycollate medium (10% wt/vol) by intraperitoneal injection. The thioglycollate medium was prepared by dissolving 0.1 g of thioglycollate powder in 100 ml distilled water, then sterilized by autoclaving. Three days after the thioglycollate injection, mice were anesthetized with ether and sacrificed by cervical dislocation. The abdominal skin was carefully opened and a 22 gauge needle was introduced into the peritoneal cavity, and 5 ml HBSS solution was injected into the peritoneal cavity with a 10 ml syringe. The abdomen was gently rubbed without removing the needle and the solution inside the peritoneal cavity was collected through the syringe. The cells were washed twice in HBSS solution and plated into tissue culture dishes (1×10^7 cells per 100 X 20 mm dish). The dishes were cultured at 37°C for 2 hours. The non-adherent cells were discarded after the culture and the adherent cells were harvested (Weaver et al., 1988). This cell preparation consisted > 95% macrophages as analyzed by FACS based on cell size and granularity.

5.2.9. Cell adhesion assay

Adhesion of macrophages to IEC-4.1 monolayers was assessed in an adhesion assay (Hauser et al., 1993). IEC-4.1 cells were grown to confluence in 96-well, flat-bottom

tissue culture plates (0.32 cm^2 culture area/well, Catalogue 25860, Corning) coated with type I collagen. The confluent epithelial monolayers were left untreated or treated with either LPS (12 ug/ml) or TNF-α (2.5 ng/ml) for various times. After washing twice in HBSS (with Ca^{2+} and Mg^{2+}), the epithelial cells were incubated with macrophages (1 x 10^5 cells/well) in DMEM supplemented with 10% FCS for 60 minutes at 37°C to allow adhesion to occur. The plates were filled with warm HBSS and washed three times after the incubation, fixed in 2% buffered glutaraldehyde, and then briefly stained with hematoxylin. The bound macrophages were counted under an inverted microscope with a grid filter (PhotoZoom™, Cambridge, MA, USA). Three random microscopic fields per well (representing a total area of 0.45 mm^2) were counted. Data were expressed as the number of bound cells per field.

In an adhesion inhibition assay, the epithelial cells were pre-incubated with blocking antibodies directed against VCAM-1 or ICAM-1 (12 ug/ml) at 37°C for 30 minutes. Macrophages (1 x 10^5) were added to the plates and incubated in the continuous presence of antibodies for 60 minutes. In some experiments, macrophages were pre-incubated with antibodies against LFA-1 or VLA-4 (12 ug/ml) for 30 minutes before co-culture with epithelial monolayers. All these antibodies used have been previously shown to block functional interactions of adhesion molecules with their ligands (Nakajima et al., 1994; Orosz et al., 1993; Isobe

et al., 1992; Harder et al., 1991). A rat anti-mouse CD4 monoclonal antibody, GK1.5, was included in each assay as the isotype control. Non-adherent cells were removed by repeated washing after the incubation. Adherent cells were counted as described above.

5.2.10. Statistics

The data are expressed as mean ± SD of triplicate assays and compared using the analysis of variance, Newman-Keuls multiple comparison test with a p value less than 0.05 as the level of significance.

5.3. RESULTS

5.3.1. Enterotoxicity of TNF-α and LPS

The direct effects of endotoxin and TNF-α, which are present at high levels during acute intestinal rejection, on IEC-4.1 cells were examined *in vitro*. In the absence of actinomycin D pre-treatment, the viability of IEC-4.1 cells was not affected by endotoxin, TNF-α, or the combination of endotoxin and TNF-α. The concentration of endotoxin examined ranged from 1.5 to 200 ug/ml and TNF-α ranged from 0.1 to 10 ng/ml. However, when IEC-4.1 cells were briefly treated with actinomycin D, a transcriptional inhibitor, TNF-α was highly cytotoxic to IEC-4.1 cells in a dose-dependent fashion. Endotoxin was unable to kill actinomycin D-treated IEC-4.1 cells, but it appeared to potentiate the cytotoxic effects of TNF-α (Figure 10).

5.3.2. Cytokine gene expression in IEC-4.1 cells in response to LPS or TNF-α

Northern blot analysis was used to examine the gene transcripts for cytokines (IL-1α, IL-1β, IL-6, TNF-α, and TGF-β) in IEC-4.1 cells. As shown in Plate 18 and 19, IEC-4.1 cells constitutively expressed IL-1α mRNA. In contrast, the unstimulated cells did not express mRNA transcripts for IL-1β, IL-6, TNF-α, and TGF-β. When IEC-4.1 cells were exposed to LPS, IL-6 and TGF-β mRNA were readily detectable. IL-6 and TGF-β mRNA peaked at 4 hours after LPS stimulation and then declined thereafter. They reached undetectable levels 8 hours after LPS stimulation. The gene transcripts for IL-1β and TNF-α were not detected in IEC-4.1 cells at any time point after LPS stimulation.

IL-1α mRNA was detected in IEC-4.1 cells cultured with or without TNF-α, and mRNA for IL-1β, IL-6, TNF-α was not detected at any time point after TNF-α stimulation. The expression of TGF-β mRNA was detected in IEC-4.1 cells at 4 hours after TNF-α stimulation (Plates 20 and 21).

5.3.3. Adhesion molecule gene expression in IEC-4.1 in response to LPS or TNF-α

VCAM-1 mRNA was readily detectable in IEC-4.1 cells by northern blot analysis whereas ICAM-1 mRNA was barely present in the absence of stimulation. When cells were exposed to LPS, expression of ICAM-1 and VCAM-1 mRNA was markedly upregulated. ICAM-1 and VCAM-1 mRNA expression displayed a different kinetics in response to LPS. ICAM-1

mRNA peaked within 4 hours after LPS stimulation and decreased to the basal level within 8 hours. VCAM-1 mRNA showed the same pattern of peak expression but decreased gradually. VCAM-1 mRNA returned to the basal level within 24 or 48 hours. The same pattern of gene expression was observed when IEC-4.1 cells were stimulated with TNF-α (Plate 22 and 23).

The expression of ICAM-1 and VCAM-1 on the surface of IEC-4.1 cells was further analyzed by FACS. The unstimulated IEC-4.1 cells expressed low levels of ICAM-1 (14%) and VCAM-1 (17%). When cells were incubated with endotoxin (12 ug/ml) for 4 hours, there was a significant upregulation of ICAM-1 and VCAM-1 expression on the cell surface. IEC-4.1 cells expressing ICAM-1 increased to 43% and cells expressing VCAM-1 increased to 92%. 24 hours after endotoxin stimulation, the expression of VCAM-1 was still maintained at high levels (93%) whereas ICAM-1 expression decreased to 31% (Figure 11). When IEC-4.1 cells were challenged with TNF-α (2.5 ng/ml), the same pattern of VCAM-1 expression was observed, but TNF-α was not as effective as LPS in the induction of ICAM-1 expression on IEC-4.1 cells (Figure 12).

5.3.4. Macrophage adhesion to IEC-4.1 monolayers

The thioglycollate-elicited peritoneal macrophages express both LFA-1 and VLA-4 as analyzed by FACS (Figure 13). Although IEC-4.1 cells constitutively express ICAM-1 and VCAM-1, the ligands of LFA-1 and VLA-4 respectively, on the cell surface, macrophage adhesion to

the unstimulated IEC-4.1 monolayer was minimal. Treatment of IEC-4.1 monolayers with LPS (12 ug/ml) induced a significant increase in macrophage adherence. The increased cell adhesion was observed 2 hours after LPS stimulation and peaked around 4 hours. Treatment of the epithelial monolayer with TNF-α (2.5 ng/ml) showed a different pattern of macrophage adhesion. A significant increase in cell adhesion started at 8 hours after TNF-α stimulation and peaked at 12 hours ($p<0.05$) (Figure 14), suggesting that LPS stimulates acute macrophage adhesion and TNF-α induces a prolonged effect.

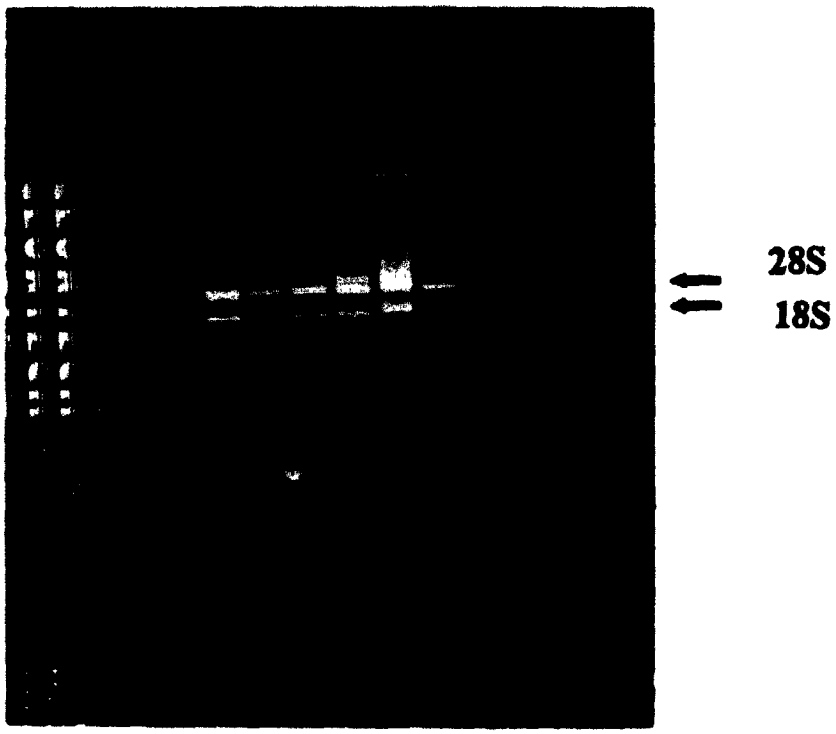
5.3.5. Adhesion molecules involved in macrophage adhesion to IEC-4.1 monolayers

Adhesion molecules involved in macrophage adhesion to IEC-4.1 monolayers were further examined using blocking monoclonal antibodies. As shown in Figure 15, LPS stimulation of IEC-4.1 cells for 8 hours induced a significant increase in macrophage adhesion ($p<0.05$) and the isotype control antibody, GK1.5, did not affect the macrophage adhesion to IEC-4.1 monolayers. However, when LPS-stimulated IEC-4.1 monolayers were pre-treated with anti-ICAM-1 antibody (12 ug/ml) and then incubated with macrophages with the continuous presence of the antibody, macrophage adhesion to the epithelial monolayer was significantly inhibited ($p<0.05$). When macrophages were pre-incubated with anti-LFA-1 antibody before co-culturing with LPS-stimulated IEC-4.1 monolayers, the inhibition of

macrophage adhesion was also significant ($p<0.05$). The anti-LFA-1 antibody was more effective than the anti-ICAM-1 antibody in blocking macrophage adhesion. Interestingly, both anti-ICAM-1 and anti-LFA-1 antibodies inhibited macrophage adhesion at a comparable level to anti-LFA-1 treatment alone.

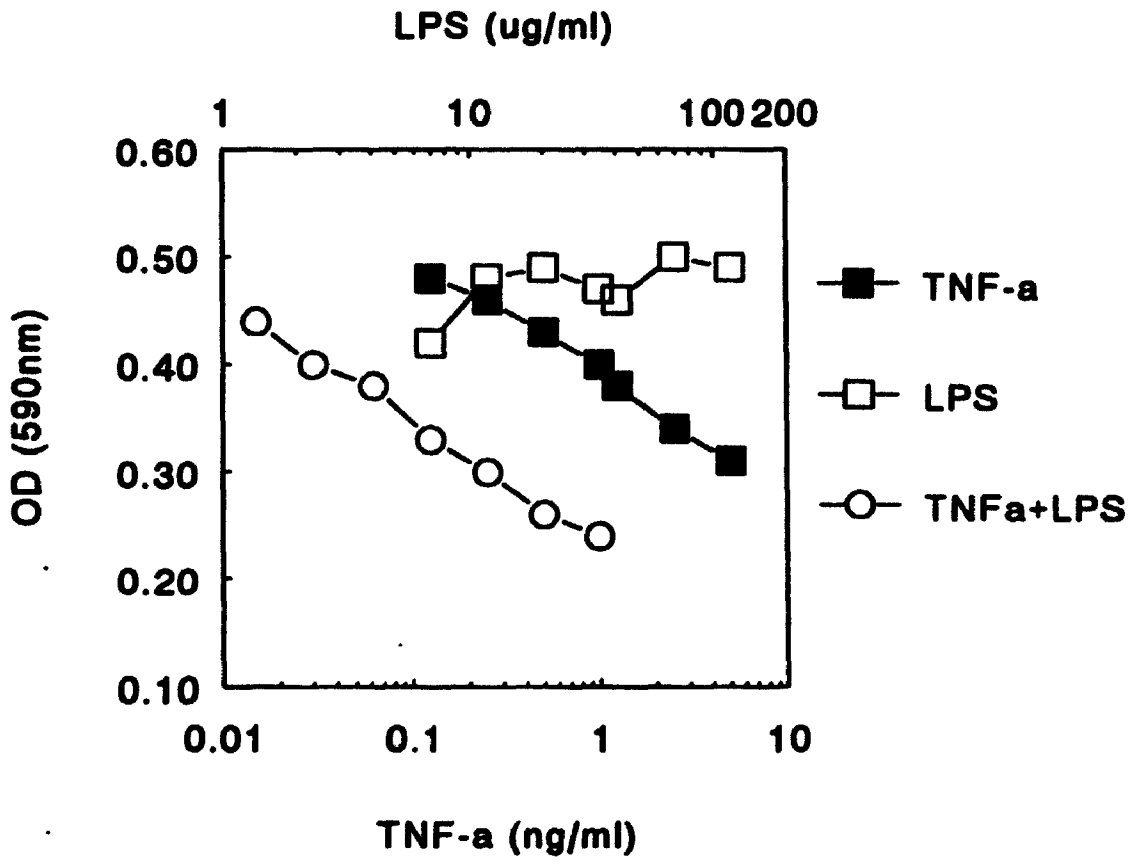
The antibody against VCAM-1 did not significantly affect macrophage adhesion to LPS-stimulated IEC-4.1 monolayers ($p>0.05$). Pre-treatment of macrophages with the antibody against VLA-4 was more effective in blocking macrophage adhesion than that of anti-VCAM-1 antibody (Figure 16). These data suggest that both ICAM-1/LFA-1 and VCAM-1/VLA-4 are involved in macrophage adhesion to LPS-stimulated IEC-4.1 cells, but ICAM-1/LFA-1 play a dominant role under the described experimental condition. When IEC-4.1 cells were stimulated with TNF-α, the same pattern of inhibition of macrophage adhesion was observed (Figures 17 and 18).

Plate 17.Structural integrity of RNA analyzed by agarose gel electrophoresis



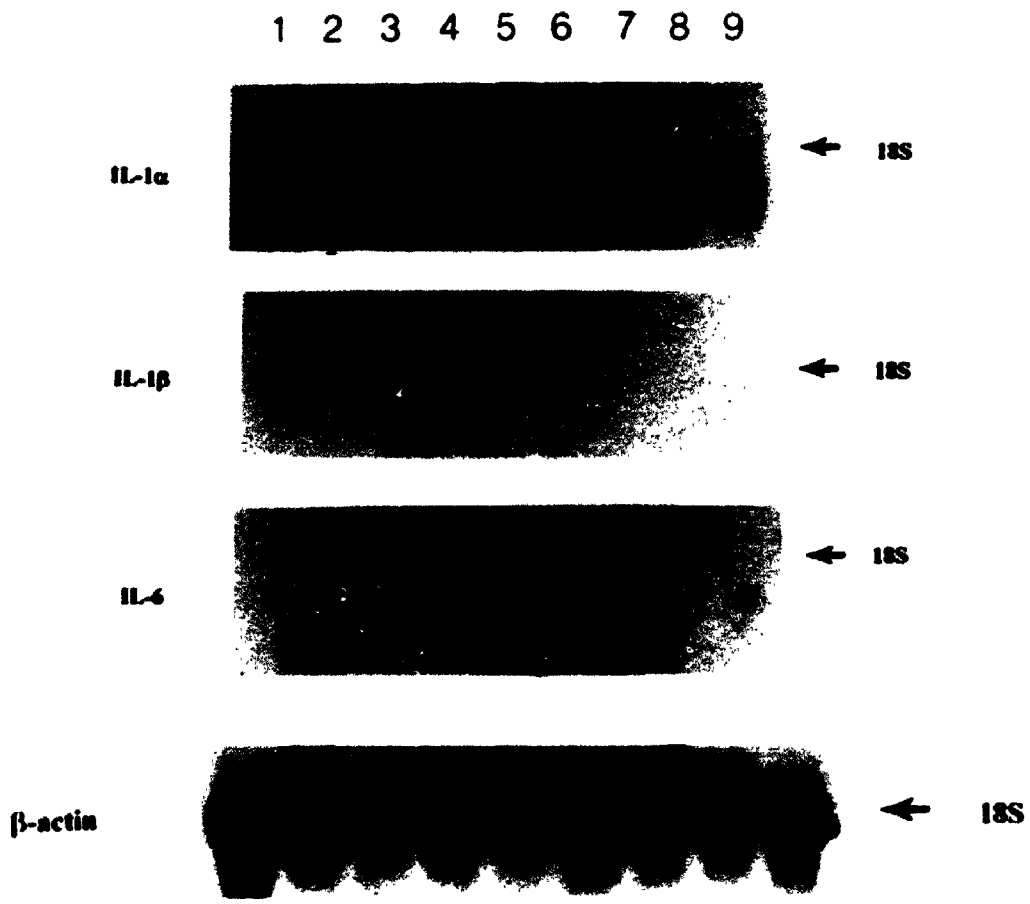
RNA from IEC-4.1 cells was electrophoresed in 1% agarose gel with ethidium bromide at 60 volts for 60 minutes and photographed under ultraviolet light. The presence of 18S and 28S ribosomal bands suggests structural integrity of RNA.

Figure 10. Enterocytotoxicity of LPS and TNF-α



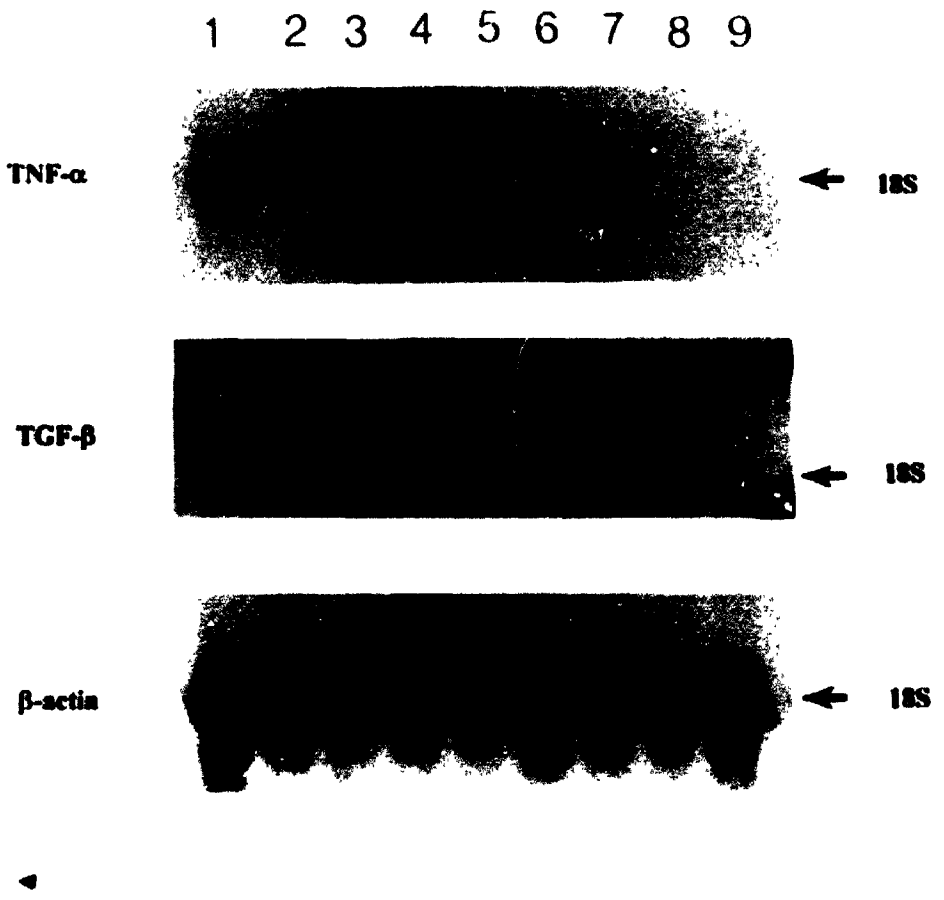
IEC-4.1 cells were briefly treated with Actinomycin D and cultured with LPS and/or TNF-α. The cell death was determined using the colorimetric assay. -□- LPS alone. -■- TNF-α alone. -O- TNF-α + LPS in which LPS was 100 ug/ml and TNF-α was diluted from 1 to 0.01 ng/ml. The top X axis is the concentration of LPS and the bottom X axis shows the concentration of TNF-α. Each point in the Figure represents the mean OD of triplicate assays.

Plate 18. IL-1α, IL-1β, and IL-6 gene expression in IEC-4.1 cells in response to LPS



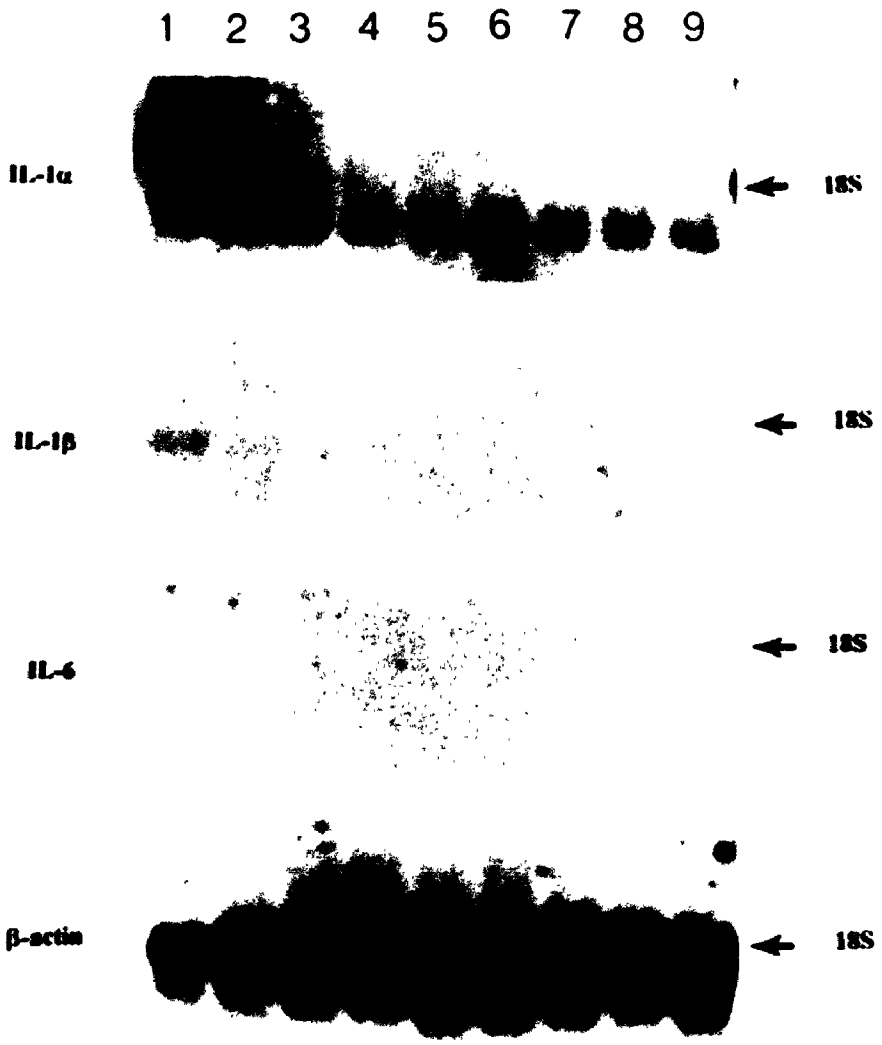
Temporal pattern of IL-1α, IL-1β, and IL-6 gene expression in IEC-4.1 cells analyzed by Northern blot analysis. Lane 1: LPS stimulated spleen cells; lane 2: unstimulated IEC-4.1 cells; lane 3 to 9: LPS stimulated IEC-4.1 cells for 1, 2, 4, 8, 12, 24, and 48 hours.

Plate 19. TNF-α and TGF-β gene expression in IEC-4.1 cells in response to LPS



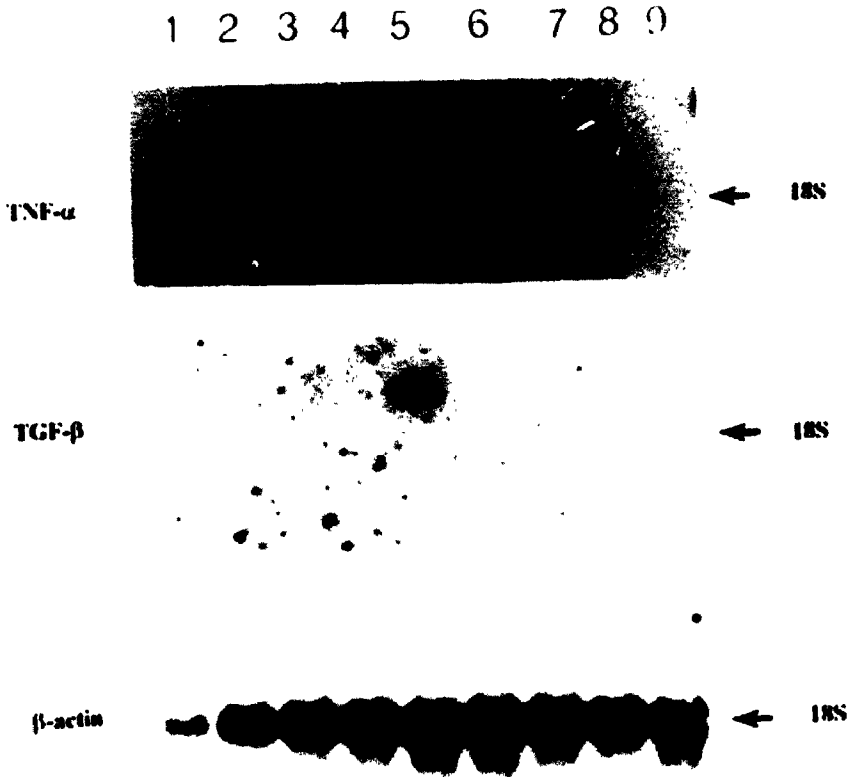
Temporal pattern of TNF-α and TGF-β gene expression in IEC-4.1 cells analyzed by Northern blot analysis. Lane 1: LPS stimulated spleen cells; lane 2: unstimulated IEC-4.1 cells; lane 3 to 9: LPS stimulated IEC-4.1 cells for 1, 2, 4, 8, 12, 24, and 48 hours.

Plate 20. IL-1α, IL-1β, and IL-6 gene expression in IEC-4.1 cells in response to TNF-α



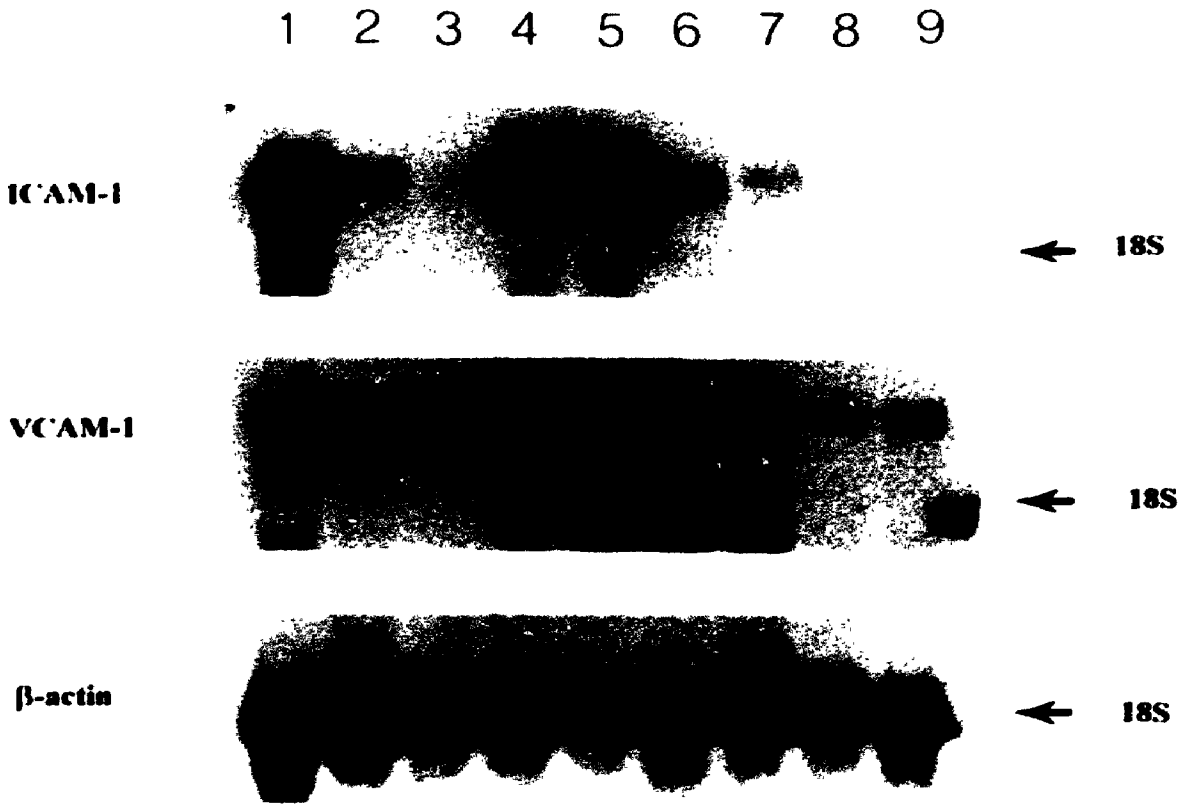
Temporal pattern of IL-1α, IL-1β, and IL-6 gene expression in IEC-4.1 cells analyzed by Northern blot analysis. Lane 1: LPS stimulated spleen cells; lane 2: unstimulated IEC-4.1 cells; lane 3 to 9: TNF-α stimulated IEC-4.1 cells for 1, 2, 4, 8, 12, 24, and 48 hours.

Plate 21. TNF-α and TGF-β gene expression in IEC-4.1 cells in response to TNF-α



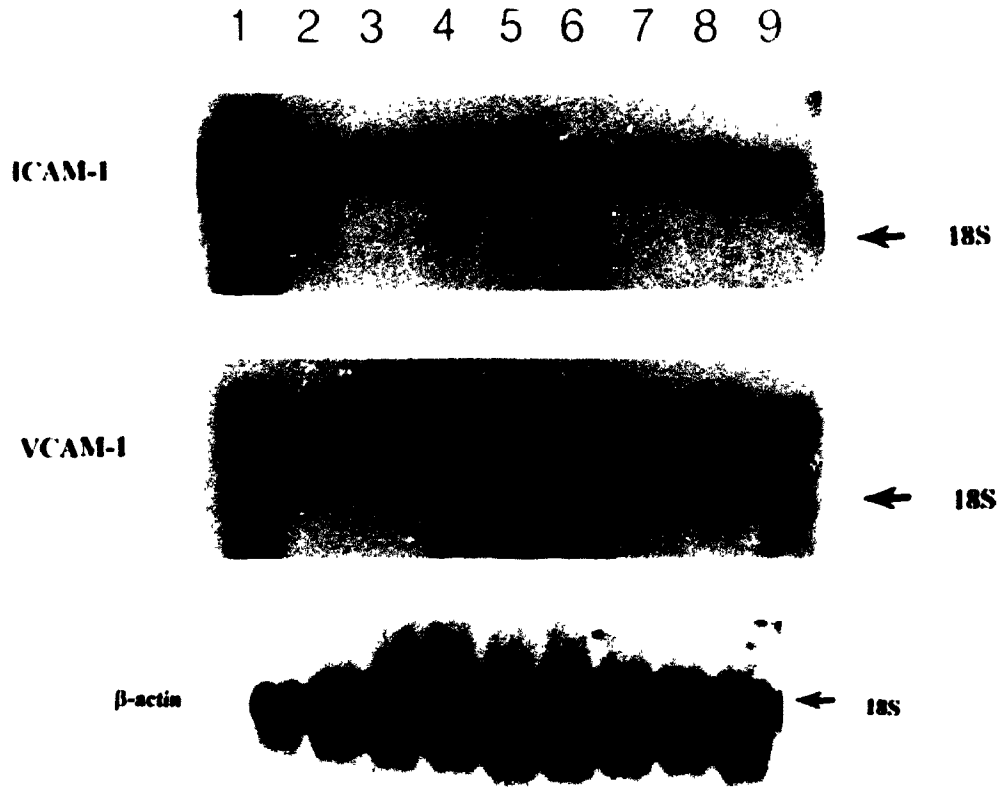
Temporal pattern of TNF-α and TGF-β gene expression in IEC-4.1 cells analyzed by Northern blot analysis. Lane 1: LPS stimulated spleen cells; lane 2: unstimulated IEC-4.1 cells; lane 3 to 9: TNF-α stimulated IEC-4.1 cells for 1, 2, 4, 8, 12, 24, and 48 hours.

Plate 22. ICAM-1 and VCAM-1 gene expression in IEC-4.1 cells in response to LPS



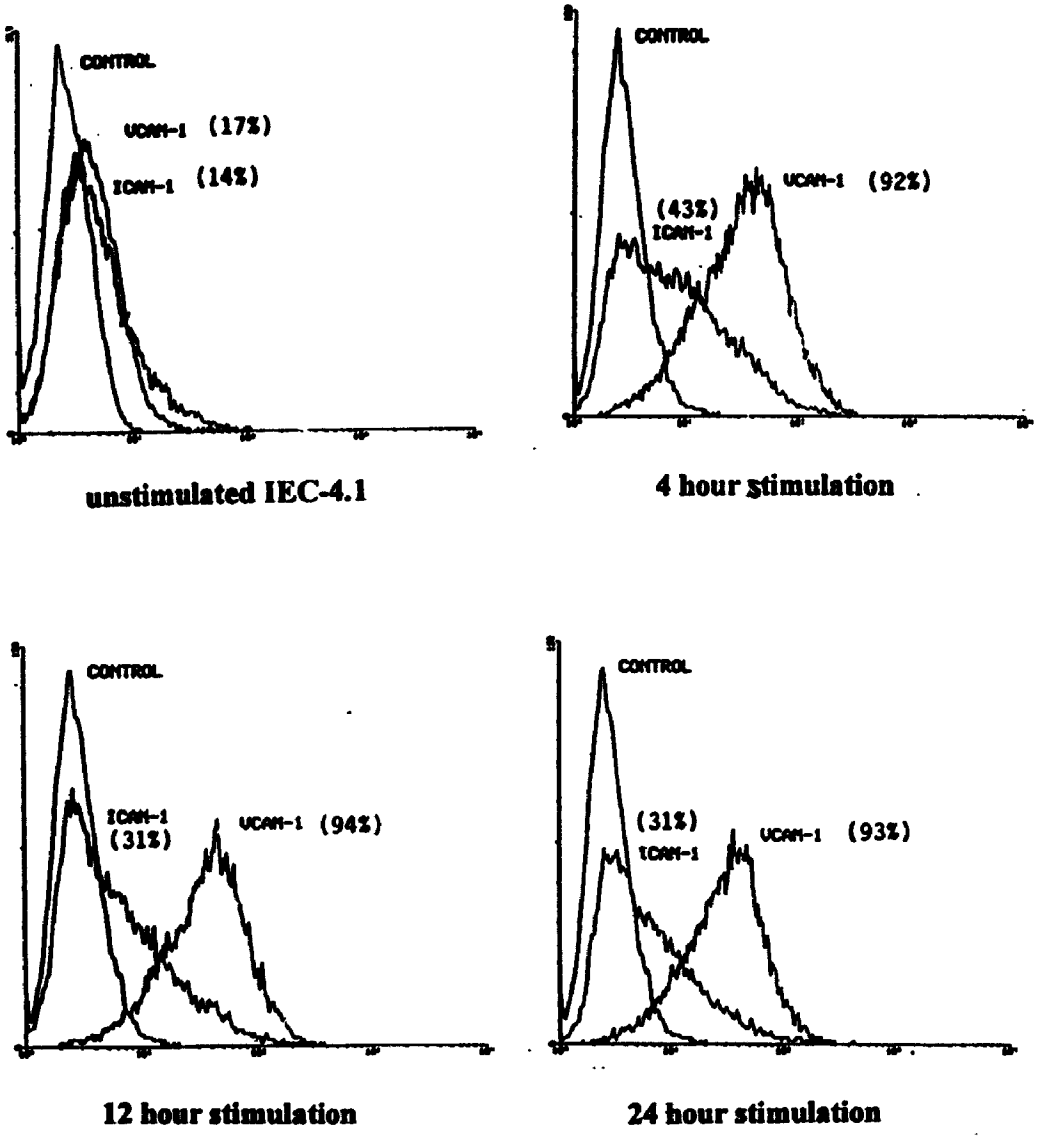
ICAM-1 and VCAM-1 gene expression by northern blot analysis. Lane 1: LPS stimulated spleen cells; lane 2: unstimulated IEC-4.1 cells; lane 3 to 9: LPS stimulated IEC-4.1 cells for 1, 2, 4, 8, 12, 24, and 48 hours.

Plate 23. ICAM-1 and VCAM-1 gene expression in IEC-4.1 cells in response to TNF-α



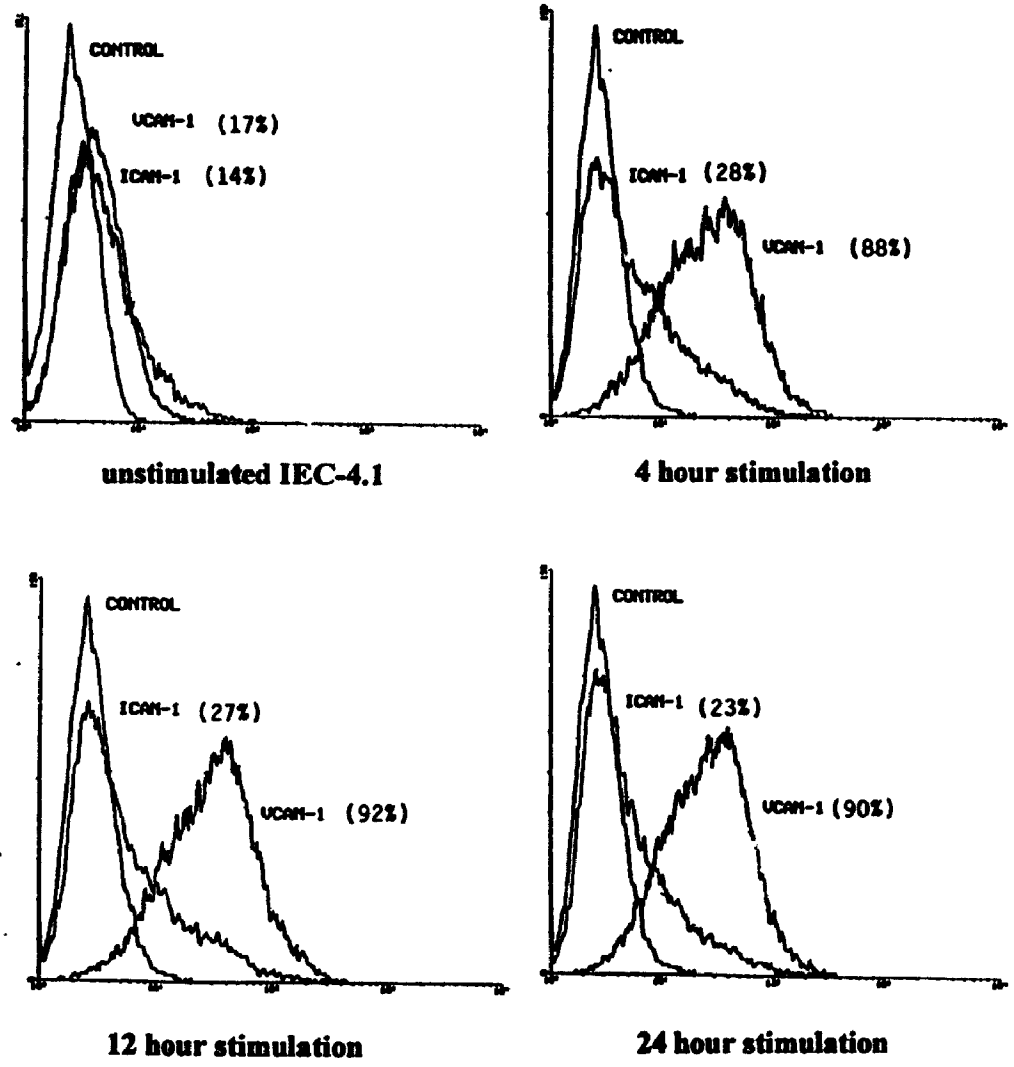
ICAM-1 and VCAM-1 gene expression by northern blot analysis. Lane 1: LPS stimulated spleen cells; lane 2: unstimulated IEC-4.1 cells; lane 3 to 9: TNF-α stimulated IEC-4.1 cells for 1, 2, 4, 8, 12, 24, and 48 hours.

Figure 11. ICAM-1 and VCAM-1 expression on LPS-stimulated IEC-4.1 cells analyzed by FACS



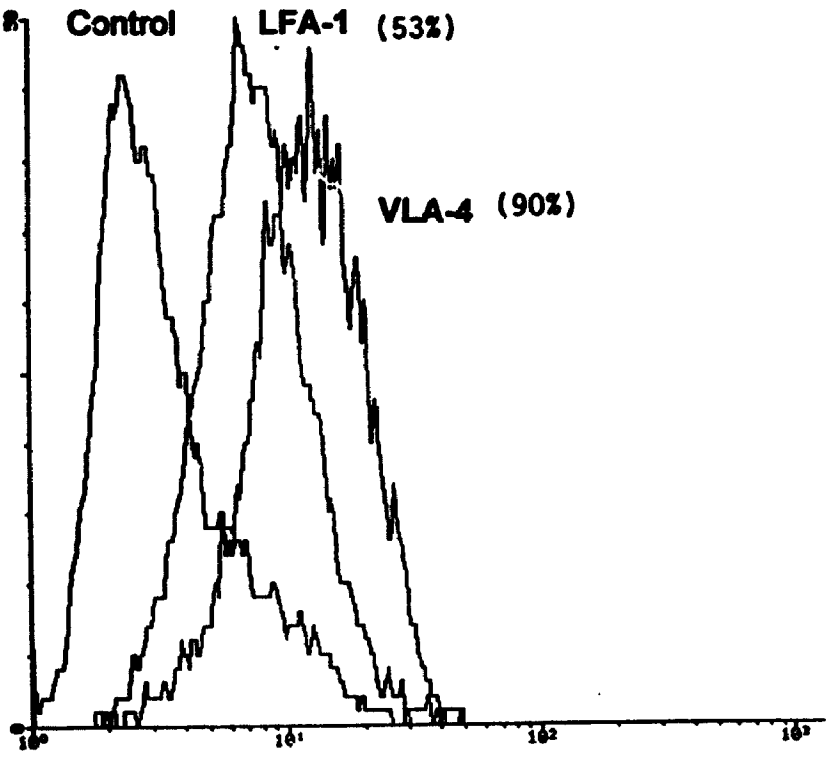
IEC-4.1 cells were stimulated with LPS (12 ug/ml) for 4, 12, or 24 hours. The stimulated cells (10^6) were stained with the rat anti-mouse ICAM-1 or VCAM-1 antibody and further stained with the FITC-conjugated goat anti-rat IgG after washing in phosphate buffered saline. Control preparations were stained with the FITC-conjugated goat anti-rat IgG alone. The fluorescence profiles generated by the staining were analyzed using FACScan and compared with the unstimulated cells. Files were collected from 2 x 10^4 cells for each sample, and data were plotted as the number of cells (Y axis) versus the log fluorescence intensity (X axis). Results are expressed as percentage of positive cells among the total number of cells counted.

Figure 12. ICAM-1 and VCAM-1 expression on TNF-α-stimulated IEC-4.1 cells analyzed by FACS



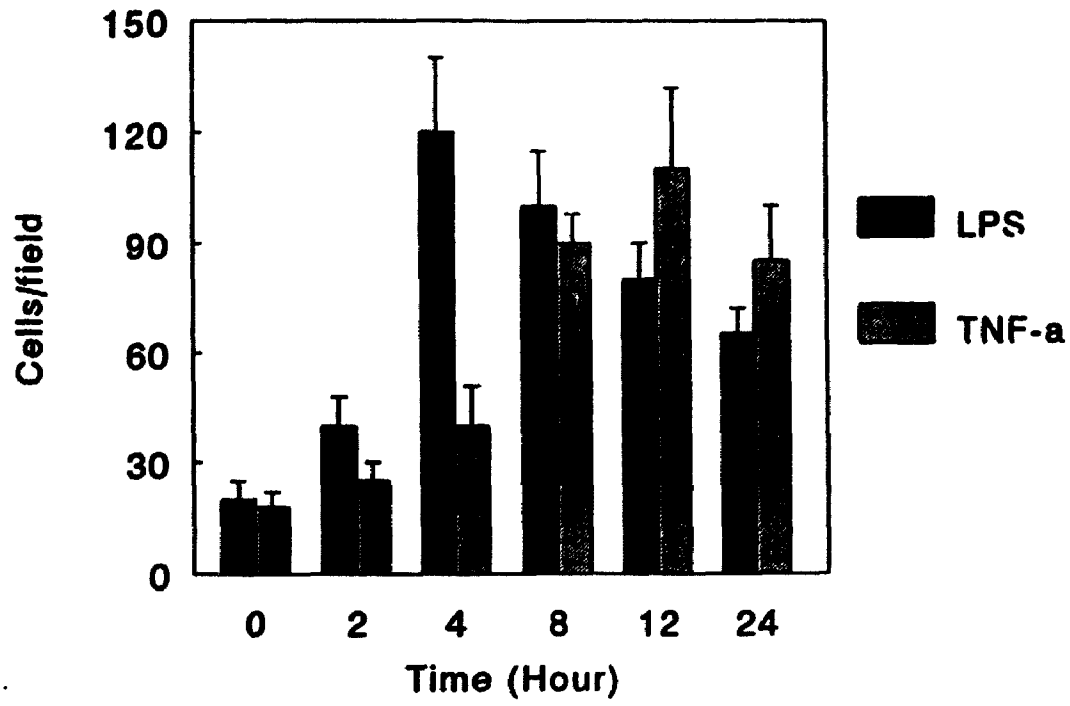
IEC-4.1 cells were stimulated with TNF-α (2.5 ng/ml) for 4, 12 or 24 hours. The stimulated cells (10^6) were stained with the rat anti-mouse ICAM-1 or VCAM-1 antibody and further stained with the FITC-conjugated goat anti-rat IgG after washing in phosphate buffered saline. Control preparations were stained with the FITC-conjugated goat anti-rat IgG alone. The fluorescence profiles generated by the staining were analyzed using FACScan and compared with the unstimulated cells. Files were collected from 2 x 10^4 cells for each sample, and data were plotted as the number of cells (Y axis) versus the log fluorescence intensity (X axis). Results are expressed as percentage of positive cells among the total number of cells counted.

Figure 13. Expression of LFA-1 and VLA-4 by macrophages analyzed by FACS



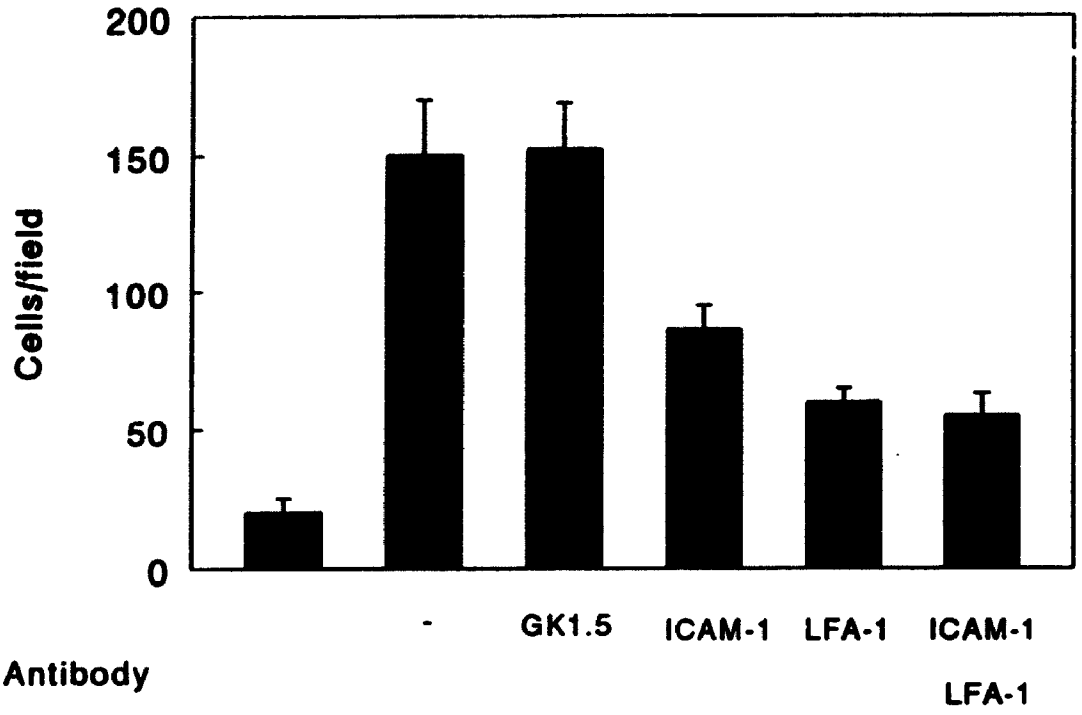
Macrophages (10^6) were stained with the rat anti-mouse LFA-1 or VLA-4 antibody and further stained with the FITC-conjugated goat anti-rat IgG after washing in phosphate buffered saline. Control preparations were stained with the FITC-conjugated goat anti-rat IgG alone. The fluorescence profiles generated by the staining were analyzed using FACScan. Files were collected from 2 x 10^4 cells for each sample, and data were plotted as the number of cells (Y axis) versus the log fluorescence intensity (X axis). Results are expressed as percentage of positive cells among the total number of cells counted.

Figure 14. Macrophage adhesion to IEC-4.1 monolayers



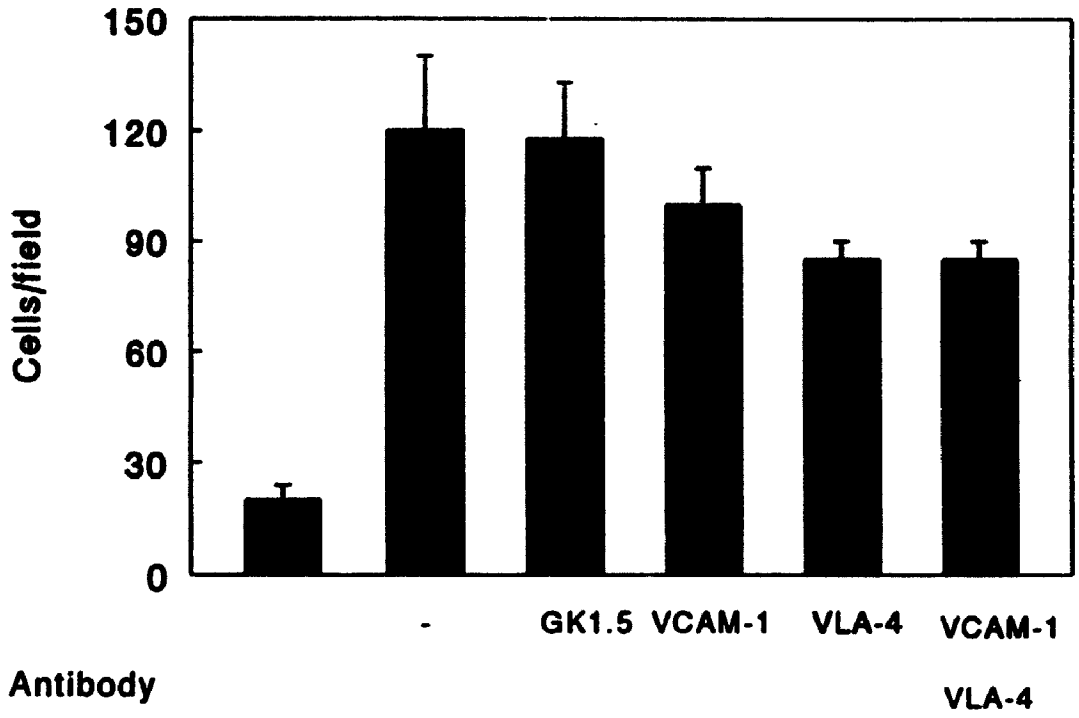
IEC-4.1 cells were pre-treated with LPS (12 ug/ml) or TNF-α (2.5 ng/ml) for 2, 4, 8, 12, 24 hours, and washed twice in HBSS before co-culturing with macrophages (1×10^5). The plates were cultured at 37°C for 60 minutes and washed again in HBSS. The bound macrophages were counted under microscope with a grid. Data are presented as the mean number of bound macrophages per field $\pm$ SD of triplicate assays.

Figure 15. ICAM-1 and LFA-1 in macrophage adhesion to LPS-stimulated IEC-4.1 cells



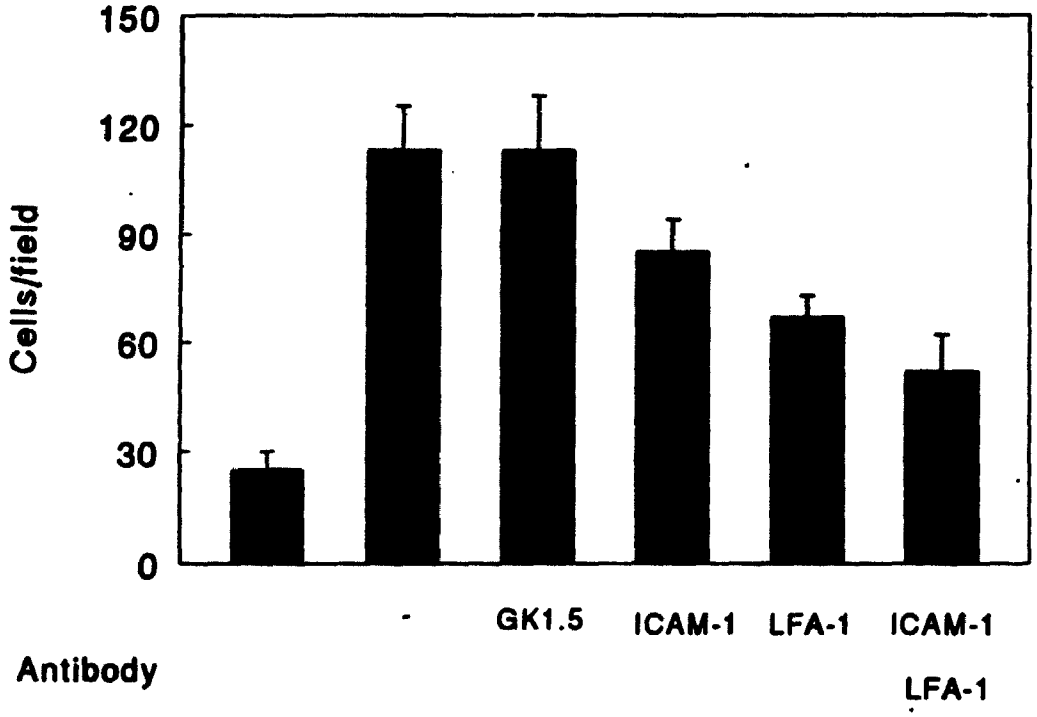
IEC-4.1 cells were pre-treated with LPS (12 ug/ml) for 8 hours and washed twice with HBSS. The IEC-4.1 monolayers were incubated with a rat anti-mouse ICAM-1 monoclonal antibody (12 ug/ml) for 30 minutes before the addition of macrophages. At the same time, macrophages were incubated with a rat anti-mouse LFA-1 antibody (12 ug/ml) for 30 minutes before being added to IEC-4.1 monolayers. GK1.5, a rat anti-mouse antibody, was used as the isotype control. Data are presented as mean $\pm$ SD of triplicate assays.

Figure 16. VCAM-1 and VLA-4 in macrophage adhesion to LPS-stimulated IEC-4.1 cells



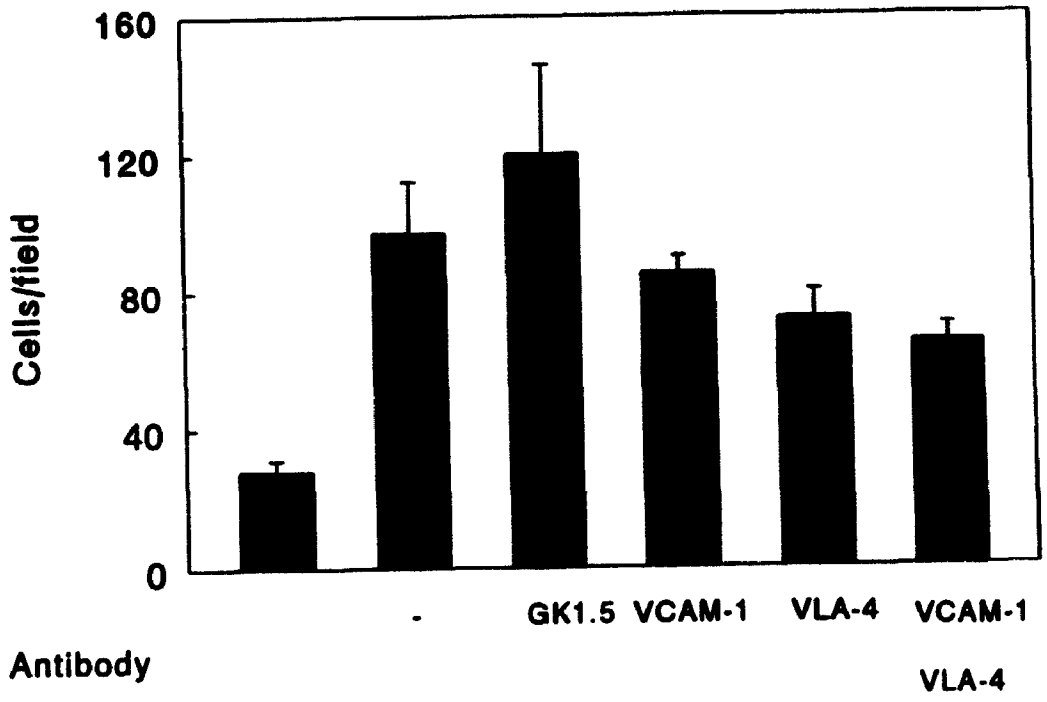
IEC-4.1 cells were pre-treated with LPS (12 ug/ml) for 8 hours and washed twice with HBSS. The IEC-4.1 monolayers were incubated with a rat anti-mouse VCAM-1 monoclonal antibody (12 ug/ml) for 30 minutes before the addition of macrophages. At the same time, macrophages were incubated with a rat anti-mouse VLA-4 antibody (12 ug/ml) for 30 minutes before being added to IEC-4.1 monolayers. GK1.5, a rat anti-mouse antibody, was used as the isotype control. Data are presented as mean $\pm$ SD of triplicate assays.

Figure 17. ICAM-1 and LFA-1 in macrophage adhesion to TNF-α-stimulated IEC-4.1 cells



IEC-4.1 cells were pre-treated with TNF-α (2.5 ng/ml) for 12 hours and washed twice with HBSS. The IEC-4.1 monolayers were incubated with a rat anti-mouse ICAM-1 monoclonal antibody (12 ug/ml) for 30 minutes before the addition of macrophages. At the same time, macrophages were incubated with a rat anti-mouse LFA-1 antibody (12 ug/ml) for 30 minutes before being added to IEC-4.1 monolayers. GK1.5, a rat anti-mouse antibody, was used as the isotype control. Data are presented as mean $\pm$ SD of triplicate assays.

Figure 18. VCAM-1 and VLA-4 in macrophage adhesion to TNF-α-stimulated IEC-4.1 cells



IEC-4.1 cells were pre-treated with TNF-α (2.5 ng/ml) for 12 hours and washed twice with HBSS. The IEC-4.1 monolayers were incubated with a rat anti-mouse VCAM-1 monoclonal antibody (12 ug/ml) for 30 minutes before the addition of macrophages. At the same time, macrophages were incubated with a rat anti-mouse VLA-4 antibody (12 ug/ml) for 30 minutes before being added to IEC-4.1 monolayers. GK1.5, a rat anti-mouse antibody, was used as the isotype control. Data are presented as mean $\pm$ SD of triplicate assays.

5.4. DISCUSSION

This study suggests that intestinal epithelial cells may participate in intestinal graft rejection through several different ways. First, intestinal epithelial cells express inflammatory cytokines (IL-1α, IL-6), and therefore can promote mucosal inflammatory responses. Second, intestinal epithelial cells express TGF-β in response to both endotoxin and TNF-α, which may interfere with the growth and differentiation of epithelial cells. Third, these cells also express adhesion molecules (ICAM-1, VCAM-1) that can effectively mediate macrophage adhesion to epithelial monolayers, and finally, intestinal epithelial cells are a vulnerable target to endotoxin and TNF-α mediated damage.

The unique gut microenvironment where epithelial cells are the first host cells to come in contact with microbial pathogens may selectively influence cytokine gene expression. IEC-4.1 cells constitutively expressed IL-1α mRNA and its expression was not affected by LPS or TNF-α. In contrast, IL-6 mRNA was undetectable in unstimulated IEC-4.1 cells, but its expression was readily induced by LPS. Interestingly, IL-1β and TNF-α mRNA were not detected by Northern blot analysis in IEC-4.1 cells cultured with or without LPS or TNF-α. This is in contrast with endothelial cells where IL-1β and TNF-α mRNA were expressed at high levels after LPS stimulation (Libby et al., 1986; Noronha et al., 1993). SV40 viral DNA transformed renal tubular epithelial cells also expressed TNF-α mRNA when stimulated

with LPS, but IL-6 mRNA was not detected under these conditions (Wuthrich et al., 1990). Recently, IL-1, IL-8, IL-10, and TNF-α gene transcripts have been reported by using epithelial cell lines derived from human colonic carcinomas, but none of these cell lines expressed IL-6 mRNA (Eckmann et al., 1993). These differences may be due to the fact that the epithelial cells used in this study are derived from small intestine whereas other studies use colonic epithelial cells. It is also possible that the carcinogenesis may alter the differentiated epithelial cell behavior of these colonic carcinomas. The selective expression of cytokines in relation to epithelial function warrants further study.

IEC-4.1 cells express differences in the expression of ICAM-1 and VCAM-1 in response to endotoxin. The gene transcripts for ICAM-1 showed early upregulation (within 4 hours) after endotoxin challenge, and then decreased sharply to the basal level. VCAM-1 mRNA had the same pattern of peak expression but the increased VCAM-1 gene transcripts persisted for a longer period of time. This difference is also reflected in the cell surface expression. FACS analysis demonstrated that the unstimulated IEC-4.1 cells expressed low levels of ICAM-1 (14%) and VCAM-1 (17%). When cells were incubated with endotoxin for 4 hours, there was a significant increase in the expression of both ICAM-1 (43%) and VCAM-1 (92%) on the cell surface. 24 hours after endotoxin stimulation, the expression of VCAM-1 was still

maintained at high levels (93%). In contrast, ICAM-1 expression decreased to 31%. Although ICAM-1 and VCAM-1 mRNA transcripts were expressed at a comparable level at 4 hours after endotoxin stimulation, the expression of VCAM-1 on the cell surface was more dramatic than that of ICAM-1. Furthermore, when IEC-4.1 cells were stimulated with endotoxin for 24 hours, VCAM-1 mRNA decreased to the basal level, but the cell surface expression of VCAM-1 was still at high levels. These data suggest that a possible post-transcriptional regulation mechanism exists for endotoxin-induced expression of ICAM-1 and VCAM-1.

The expression of ICAM-1 and VCAM-1 on IEC-4.1 cells can effectively mediate macrophage adhesion. This finding may explain the predominance of macrophages in the intestinal epithelium during acute intestinal rejection. It appears that macrophage adhesion to endotoxin or TNF-α stimulated IEC-4.1 cells requires both LFA-1/ICAM-1 and VLA-4/VCAM-1 since antibodies against either LFA-1 or VLA-4 significantly inhibit macrophage adhesion to the epithelial monolayers. LFA-1/ICAM-1 seems to play a dominant role in mediating macrophage adhesion to stimulated IEC-4.1 monolayers as the antibody against LFA-1 blocks macrophage adhesion more effectively. In the adhesion inhibition assay, we found that antibodies against ICAM-1 or VCAM-1 on epithelial cells were not as effective as those against LFA-1 or VLA-4 in blocking macrophage adhesion, suggesting that LFA-1 or VLA-4 expressed on macrophages may also bind to ligands on

epithelial cells other than ICAM-1 or VCAM-1. It has been shown that LFA-1 also binds to ICAM-2 or ICAM-3, and VLA-4 can also bind to a matrix protein fibronectin (Pulido et al., 1991). Further studies are required to define whether IEC-4.1 cells also express ICAM-2, or ICAM-3, and to examine whether an additional ligand pair such as ELAM-1/sLex play a role in macrophage adhesion.

Leukocyte migration across intestinal epithelium occurs in the basolateral-to-apical direction *in vivo*. Therefore, it is likely that the adhesive interactions between macrophages and epithelial cells take place in the basolateral region of epithelial cells. Recent studies have demonstrated that neutrophil transepithelial migration exists at both basolateral-to-apical and apical-to-basolateral directions, although migration in the physiological direction is quantitatively greater than that in the reverse direction (Nash et al., 1987; Parkos et al., 1991). Such data suggest that the initial binding sites are also present in the apical membrane of epithelial cells but that the availability of these sites is limited in the apical as opposed to the basolateral membrane. In the present study, we demonstrate that endotoxin and TNF-α can effectively promote macrophage adhesion to the apical membrane of IEC-4.1 cells, suggesting that the expression of adhesion molecules at the apical surface of epithelial cells can be modulated by endotoxin and cytokines. Furthermore, the translocated macrophages can interact with the apical

domain of epithelial cells via adhesion molecules. The relatively high concentration of endotoxin and N-formyl-peptides that occupy the intestinal lumen are potent activators of macrophages. When activated, macrophages are capable of modulating the fundamental characteristics of epithelial structure and function through such interactions.

This study also suggests that several mechanisms may contribute to epithelial cell damage during acute intestinal rejection. Acute intestinal rejection is associated with increased production of TNF-α. We found that TNF-α was cytotoxic to epithelial cells. Interestingly, the cytotoxic effect of TNF-α was evident only when IEC-4.1 cells were pre-treated with actinomycin D, a transcriptional inhibitor, indicating that a protective factor(s) is also induced when IEC-4.1 cells are exposed to TNF-α, and the determinant of the action of TNF-α on IEC-4.1 cells is the balance of detrimental versus protective mechanisms. Previous studies have shown that the induction of mitochondrial manganese superoxide dismutase (Wong et al., 1988), heat shock proteins (Jaattela et al., 1992), and the antiprotease plasminogen activator inhibitor type II (Medcalf et al., 1988) are among the protective effects evoked by TNF-α. It is likely that IEC-4.1 cells are susceptible to TNF-α-mediated toxicity when the transcription of the protective factors was inhibited. Although endotoxin itself did not affect the viability of IEC-4.1 cells, it did potentiate the killing of IEC-4.1 cells mediated by TNF-α.

The increased expression of TGF-β by epithelial cells in response to endotoxin or TNF-α may disrupt the dynamic balance between epithelial cell renewal and differentiation. Intestinal epithelial cells are in a state of rapid proliferation and differentiation along the crypt-villus axis in a finely tuned fashion and this process is regulated, at least in part, by growth factors such as TGF-α and TGF-β (Koyama et al., 1989; Lin et al., 1993). TGF-β is expressed as epithelial cells migrate from the crypt to villus tips, suggesting that TGF-β may have a role in cell differentiation. TGF-β also inhibits cell proliferation by arresting cells in G1 phase (Lin et al., 1993). TGF-β in conjunction with endotoxin and/or TNF-α may be responsible for the altered epithelial architecture and focal crypt necrosis observed in acute intestinal rejection.

TGF-β is a central mediator in wound healing and in epithelial cell restitution following *in vitro* epithelial cell damage (Lin et al., 1993). TGF-β stimulates proliferation of fibroblasts and induces collagen synthesis by fibroblasts and smooth muscle cells (Lin et al., 1993). The increased synthesis of extracellular matrix stimulated by TGF-β may disrupt the basement membrane that underlies the intestinal epithelium. Since the structure and function of intestinal villi are highly dependent on the integrity of the basement membrane, the increased extracellular matrix synthesis is likely to have profound effects on epithelial structure.

The increased expression of adhesion molecules on the surface of epithelial cells in response to endotoxin or TNF-α may promote macrophage adhesion to epithelial cells and the subsequent accumulation of macrophages in the intestinal epithelium. The macrophages, which are frequently present in intestinal mucosa during intestinal rejection, have the potential to produce a number of factors that are important in the development of epithelial lesions.

Our data suggest that endotoxin-targeted therapy and selective anti-cytokine therapy may reduce the risk of intestinal epithelial damage during graft rejection. Techniques to minimize the effects of endotoxin include treatment with anti-endotoxin antibodies and non-toxic LPS (Loppnow et al., 1990). Strategies to block the effects of cytokines include monoclonal antibodies and receptor antagonists to the inflammatory cytokines (Imagawa et al., 1990; Bolling et al., 1992; Maury et al., 1987), as well as new methods of specific transcriptional and translational interference. Furthermore, antibodies directed against adhesion molecules, particularly ICAM-1/LFA-1, may help to prevent accumulation of macrophages in the intestinal epithelium and the subsequent epithelial damage.

CHAPTER 6. CONCLUSIONS

Significant progress has been made in the past few years in the field of IT, and today we are beginning to have some long-term survivors following clinical IT (McAlister et al., 1992). Although more powerful immunosuppressive agents such as cyclosporin A and FK506 have brought us closer to the therapeutic potentials of IT, graft rejection still remains the major challenge. The need remains for a better understanding and more effective management of intestinal rejection.

In this project, the endotoxin and inflammatory cytokines in acute intestinal allograft rejection and their roles in the pathogenesis of epithelial cell damage were examined. The original contributions of this project are summarized below.

1. The epithelial damage during acute intestinal rejection is associated with elevated levels of endotoxin and TNF-α in the peripheral blood.

Intestinal rejection appears to take two different but interrelated pathways. The first is initiated by MHC antigens through the activation of allogeneic T cells; this process occurs early and is antigen specific. The other is the inflammatory response initiated by luminal bacteria-derived endotoxin; this process is antigen non-specific and probably serves as an amplification loop of accelerated epithelial damage. The initial allogeneic T cell activation

appears to be a primary event that occurs in the graft MLN and PP prior to any signs of histological rejection, and the intestinal mucosa is often spared at this moment. As rejection progresses, the intestinal mucosa, particularly the lamina propria, becomes actively inflamed and the epithelial architecture is altered at this time. In untreated animal models, the time from altered epithelial cell growth to total epithelial sloughing is very short, ranging from 2 to 3 days. This process is accompanied by increased gut permeability, bacterial translocation, and the subsequent release of endotoxin, which in turn promotes mucosal inflammation and epithelial damage through the activation of infiltrating macrophages, blood vessel endothelial cells, and intestinal epithelial cells. Endotoxin and TNF-α stimulate the expression of adhesion molecules and inflammatory cytokines by intestinal epithelial cells, which promotes macrophage adhesion and migration into the intestinal epithelium. Endotoxin is also a potent chemoattractant to macrophages and neutrophils. These observations may explain the predominance of these cell types in the intestinal epithelium during acute intestinal rejection. The primary target of endotoxin-mediated damage appears to be the intestinal epithelium, since the elevated levels of endotoxin and TNF-α in the peripheral blood correlated well with the progressive epithelial damage.

2. The optimal conditions for epithelial isolation and primary epithelial culture are established, which allows for the initial study of epithelial cells and successful cell transfection.

3. Multiple epithelial cell lines (seven) from the small intestine of BALB/c mice are established and one epithelial cell clone (IEC-4.1) is subsequently characterized.

IEC-4.1 cells form confluent monolayers cultured in K1 medium, and express well-developed microvilli, junctional protein ZO-1, MHC class I, and low levels of MHC class II molecules. IEC-4.1 cells also express adhesion molecules (ICAM-1 and VCAM-1) in response to endotoxin or TNF-α, which can effectively promote macrophage adhesion. Thus, IEC-4.1 cells may be a valuable model to study the immunology of epithelial cells and the epithelial-leukocyte interactions.

4. The epithelial cells of the small intestine express inflammatory cytokines and adhesion molecules, two fundamental events of regulating leukocyte trafficking, and therefore, they may be the active participants in mucosal inflammation.

Small intestinal epithelial cells that line the intestinal tract are viewed predominantly as absorptive and secretory cells. Accumulating evidence suggests that the intestinal epithelial cells can express soluble mediators known to be important in the communication between inflammatory cells and cells of the immune system. We

demonstrate that IEC-4.1 cells express IL-1α mRNA, and also express IL-6 and TGF-β mRNA in response to endotoxin that is present at high levels during acute intestinal rejection. These findings, coupled with the expression of adhesion molecules, either constitutively or after inflammatory stimulation, point to an important role of intestinal epithelial cells as an essential component of mucosal inflammation.

The experimental models established in this project will also serve as a foundation for further study of the physiology and the immunology of intestinal epithelial cells.

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