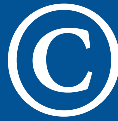

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Effects of 5-Fluorouracil on Oral Barrier Functions

INGER VON BÜLTZINGSLÖWEN

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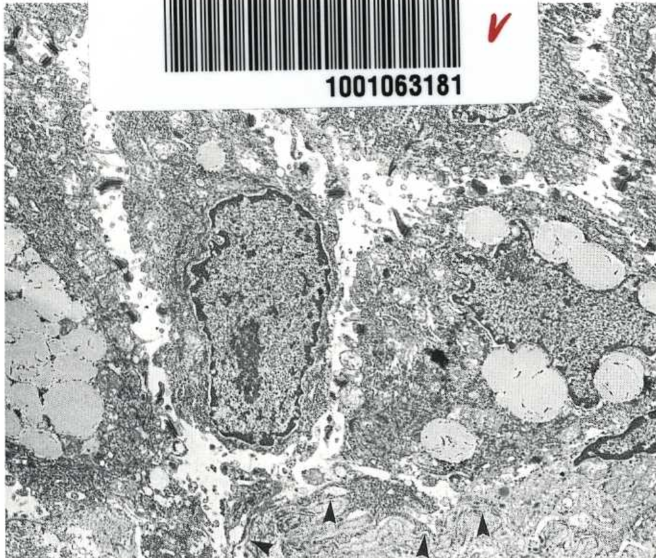

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Akademisk avhandling

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I. 5-Fluorouracil induces autophagic degeneration in rat oral keratinocytes. I. von Bültzingslöwen, M. Jontell, P. Hurst, U. Nannmark, T. Kardos Oral Oncol. 2001 Sep;37(6):537-44.

II. Macrophages, dendritic cells and T lymphocytes in rat buccal mucosa and dental pulp following 5-fluorouracil treatment. I. von Bültzingslöwen, M. Jontell Eur J Oral Sci. 1999 Jun;107(3):194-201.

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IV. Microflora of the oral cavity and intestine in 5-fluorouracil treated rats, bacterial translocation to cervical and mesenteric lymph nodes and effects of probiotic bacteria. I. v. Bültzingslöwen, I. Adlerbert, A. Wold, G. Dahlén, M. Jontell Submitted to Oral Microbiol Immunol.

Abstract

EFFECTS OF 5-FLUOROURACIL ON ORAL BARRIER FUNCTIONS

Inger von Bültzingslöwen

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Many anticancer drugs, e.g. 5-fluorouracil (5-FU), may cause oral mucositis and ulcerations. These adverse reactions can be severe and debilitating to the patient, and adjustment of the cancer treatment may be necessary. Efforts to develop reliable clinical protocols to relieve the oral side effects have so far been disappointing. Thus, further knowledge regarding the pathophysiology behind these lesions is warranted.

This thesis focused on some influences of 5-FU on major oral barrier functions, the oral epithelium, the local immune defence and the microflora.

Rats were treated with 5-FU (30 mg/kg; 50 mg/kg) *i.v.* In one experiment, the probiotic bacteria *Lactobacillus plantarum* 299v, was delivered in the drinking water during 5-FU treatment, to modify bacterial overgrowth.

After the animals were sacrificed, biopsies were taken. Oral keratinocytes were investigated for 5-FU induced mode of cell death. Analysis was performed by flow cytometry, vital dye exclusion test, the TUNEL method and ultrastructural analysis. The number of local immunocompetent cells of the oral mucosa was compared with the number of similar cell populations of the dental pulp. MHC class II molecule expressing cells of the buccal epithelium and dental pulp were assessed for the capacity to induce a ConA stimulated T cell proliferation. Changes in bacterial homeostasis of the oral cavity and intestine were evaluated and predominating groups of facultative anaerobes were identified by colony morphology and gram staining appearance. The cervical and mesenteric lymph nodes were analysed for any numbers of viable bacteria.

5-FU treatment caused alterations in the keratinocytes consistent with autophagic degeneration. The local cellular immune defence of the oral mucosa and dental pulp was affected. 5-FU caused an increase in the total number of bacteria and the number of facultative anaerobes in the oral cavity and in the number of facultative anaerobes in the intestine. The proportions of facultative gram-negative rods increased. Bacteria increased in numbers in both the cervical and mesenteric lymph nodes. These findings reinforce the oral cavity, along with the gastrointestinal tract, as an important source for bacterial dissemination. *L. plantarum* 299v did to some extent normalise 5-FU induced disturbances in the oral and intestinal microbiota and improve the well-being of the animals.

Conclusions: Influences of 5-FU on oral barrier functions were demonstrated. 5-FU may disrupt the oral epithelium, decrease the immune response and disturb the microflora. The findings indicate that the cervical lymph nodes may be an important route for bacterial dissemination from the oral cavity. Probiotic bacteria may have a positive effect on some of these functions.

Key words: 5-FU, rat, adverse effects, autophagic degeneration, immune system, microflora, mouth.

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Effects of 5-Fluorouracil on Oral Barrier Functions

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2002

COVER ILLUSTRATION:

Keratinocytes of the basal layer of the buccal epithelium, showing degenerative features after 5-fluorouracil treatment *in vivo*

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PREFACE

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. 5-Fluorouracil induces autophagic degeneration in rat oral keratinocytes

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I. v. Bültzingslöwen, I. Adlerbert, A. Wold, G. Dahlén, M. Jontell

Submitted to Oral Microbiol Immunol

ABBREVIATIONS

5-FU	5-fluorouracil
5-FU+30	30 mg/kg 5-fluorouracil
5-FU+50	50 mg/kg 5-fluorouracil
bp	base pair
BSA	bovine serum albumin
CD	cluster of differentiation
CFU	colony forming units
Con A	concanavalin A
CTL	cytotoxic T lymphocyte
DAB	3,3´-diaminobenzidine-tetrahydrochloride
DC	dendritic cell
DMEM	Dulbecco´s modified Eagle´medium
DMEM+	DMEM supplemented with 2 mmol/L glutamine and 20 mg/L gentamicin
DMEM++	DMEM+ supplemented with 20% fetal calf serum
DPD	dihydropyrimidine dehydrogenase
dTTP	deoxythymidin triphosphate
dUTP	deoxyuridine triphosphate
ED2	a differentiation-associated antigen present on tissue-resident macrophages
EDTA	ethylene diamine tetraacetic acid
FACS	fluorescence activated cell sorter
FdUMP	5-fluoro-deoxyuridine monophosphate
FITC	fluorescein isothiocyanate
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
Ig	immunoglobulin
IL	interleukin
i.v.	intravenously
L. plantarum	*Lactobacillus plantarum*
LC	Langerhans cell
LPS	lipopolysaccaride

M	molar
MHC	major histocompatibility complex
n	number
NK cells	natural killer cells
OX-6	monoclonal atibody against rat MHC class II
OX-34	monoclonal atibody against rat CD2 molecule
PBS	phosphate buffered saline
S.D.	standard deviation
TBS	Tris-HCL buffer (0.05 M; pH 7.6) containing 0.9% NaCl
TBS+	TBS supplemented with bovine serum albumin
TCR	T cell receptor
T_H	helper T cell
TUNEL	terminal deoxinucleotydyll tarnsferase (TdT)-mediated deoxuyridine triphosphate (dUTP) biotin nick end labeling
WBC	white blood cells
VMG	viability medium Gothenburg

INTRODUCTION

Many anticancer drugs have a low therapeutic index and may cause severe side effects in the oral cavity. The oral side effects are reflected in inflammation and ulcerations of the oral mucosa, bleeding, xerostomia and local or disseminated infections. Adverse responses of a similar nature may also develop in the pharynx and in the gastrointestinal tract. Patients medicated with anticancer drugs may sometimes suffer from oral side effects to the degree that makes it necessary to adjust the course of cancer treatment. Improved methods to accommodate the adverse oral effects are much in need.

Comprehensive clinical investigations have been performed with different agents in efforts to find means to modify the oral adverse effects. However, the pathophysiology of the conditions largely remains to be defined (Sonis 1998). In order to develop new treatment modalities, further basic knowledge is warranted regarding what cellular events that cytotoxic drugs induce in the oral cavity.

The various groups of anticancer drugs affect cells at different levels in the cell cycle, with different outcomes. It is therefore of great value, if possible to study one anticancer drug at a time. Results from studies involving a mixture of antineoplastic agents are not necessarily applicable to all anticancer drugs.

One group of antineoplastic agents specifically known to cause oral side effects, is antimetabolites. 5-Fluorouracil (5-FU) is an antimetabolite that has been extensively utilised for over thirty years (Heidelberg 1957) in the treatment of a wide range of solid tumours, in the gastrointestinal tract, breast and head and neck. 5-FU induces mucosal toxicity that may sometimes be severe and lifethreatening. For this reason 5-FU was selected for the investigations that form the basis for this thesis.

AIMS OF THE THESIS

The long-term objectives of my research are to elucidate the effects of cytotoxic drugs, with major dose limiting side effects, on oral barrier functions.

This thesis focused on effects of 5-FU on the *oral epithelium*, *local immune defence* and *microbial homeostasis*, major barrier functions of the oral cavity.

The following specific questions were formulated.

- Does 5-FU treatment induce an apoptotic mode of cell death in the keratinocytes of the epithelium of oral buccal mucosa? (I)

- Do the numbers of macrophages, dendritic cells (DC) and T lymphocytes, cells of the cellular immune defence in the oral buccal mucosa, become affected by 5-FU and how is the effect on the number of these cells in the buccal mucosa, compared to the dental pulp, a tissue not normally exposed to microbial challenges? (II)

- Does 5-FU influence the capacity of oral soft tissue DC to induce T cell proliferation? (III)

- How is the equilibrium of the bacterial population of the oral cavity and the intestine affected by 5-FU treatment? (IV)

- Are there signs of bacterial translocation to cervical lymph nodes in 5-FU treated rats and how is bacterial translocation to the cervical lymph nodes compared to the mesenteric lymph nodes? (IV)

- Can the probiotic bacteria *Lactobacillus plantarum* 299v normalise 5-FU induced disturbances in the microbiota and prevent bacterial dissemination to lymph nodes? (IV)

- Can *Lactobacillus plantarum* 299v improve the well being of the rats, measured by rat body weight, amount of ingested food and degree of diarrhoea? (IV)

BACKGROUND

The body endeavours to maintain homeostasis, a tendency to uniformity and stability in the normal body states. In order to maintain homeostasis, *barrier functions* are in effect to protect the internal environment and to prevent the body from emitting essential matters. An exchange with the external environment is also a fundamental part of homeostasis. Thus, the barriers of the body must have selective functions.

The *epithelium* and the *immune system* constitute selective barrier functions in the oropharyngeal and gastrointestinal tract as does the *bacterial equilibrium* of these body sites. If one or more of the barrier functions are affected, this may lead to a distorted homeostasis that may give rise to severe local or generalised adverse effects.

Cytotoxic drugs affect normal cells with a high turnover rate as well as malignant cells. Thus, tissues that are constantly renewed are likely to be affected by antineoplastic agents. In the oral cavity both cells of the epithelium and many cells of the immune system, and bacteria have a high turnover rate.

ANTICANCER DRUGS

Tumours originate from subversion of the processes that control normal cell growth, location and mortality. A loss of normal control mechanisms in tumour cells arises from gene mutations (Hesketh 1997).

Cancer chemotherapy aims at killing tumour cells. The main objective of the drug therapy is to induce cell damages, leading to the activation of cell death mechanisms. Anticancer drugs can be classified according to functions into four different groups, namely *alkylating agents*, *topoisomerase inhibitors*, *antimetabolites* and *mitosis inhibitors* (Hansson *et al.* 1998).

Alkylating agents. Alkylating agents bind to one or two bases in the DNA molecule. The ones binding to two bases are those of most clinical interest. They cause intrastrand cross-links between bases of the same DNA strand in a DNA molecule, or interstrand cross-links between

bases of the complementary DNA strain in a DNA molecule. If the cell is not capable of DNA repair it will die.

Topoisomeras inhibitors. Topoisomeras inhibitors stabilise naturally occurring strand breaks, enzymatically induced by topoisomerases. Normally such strand breaks would be repaired. If the cell is not able to repair its DNA, it will not survive.

Antimetabolites. Antimetabolites are synthetic compounds, usually a structural analogue of a normal metabolite, which interferes with the utilisation of the metabolite to which it is structurally related. Antimetabolites cause DNA strand breaks by incorporating distorted molecules in the DNA. If the cell cannot repair the damaged DNA molecule it will die, given that the cell's normal intrinsic cell death program works. Antimetabolites are classified into antifolates, (e.g. methotrexate), purine antimetabolites, (e.g. azatioprin, merkaptopurin) and fluoropyrimidines (e.g. fluorouracil).

Mitosis inhibitors interact with cell tubulin, which is normally polymerised to microtubuli. Microtubuli is a component of the cell cytosceleton and are involved in chromosome movements during mitosis. Mitosis inhibitors induce cytotoxicity by interacting with tubulin.

5-FLUOROURACIL (5-FU)

5-Fluorouracil (5-FU) is biochemically the simplest of the fluorinated pyrimidines. This cytotoxic drug is an analogue to the naturally occuring pyrimidine uracil with a fluorine atom at the 5 position of the pyrimidine ring, instead of hydrogen. 5-FU is administered *i.v.*, as bolus injections or as continuous infusions. In recent years, it has also been used as a prodrug *per os*. 5-FU is non-toxic before it is metabolised intracellularly. The drug enters the cells very rapidly by facilitated diffusion (temperature- and energy-independant) through the same pathway as uracil (Wohlhueter *et al.* 1980).

Anabolic pathways

5-FU may cause cell injury by two major anabolic pathways:

1. inhibition of the enzyme thymidylate synthase (TS) leading to impaired DNA formation
2. incorporation into RNA and growth inhibition

1. Inhibition of the enzyme thymidylate synthase (TS) and impaired DNA formation

A single DNA strand, a polynucleotide, is constructed from a series of phosphodiester-linked sugar residues, nucleotides, each carrying a base. The bases are the purines adenine (A) and guanine (G), and the pyrimidines cytosine (C) and thymine (T). Purine and pyrimidine bases are linked together in a double stranded DNA (e.g. A-T; G-C) (Mathews and van Holde 1996).

When uracil has entered the cell it will participate in the formation of new DNA. It will bind to deoxyribose and in that way the nucleoside deoxyuridine (dUrd) is formed. When 5-fluoro-uracil is administered, fluoro-deoxyuridine (FdUrd) will be formed instead. The nucleosides are converted to nucleotides by thymidine kinase by linking a phosphate group to the nucleoside. The nucleotide with a uracil base is termed deoxyuridinemonophosphate, dUMP. If the uracil is replaced by 5-fluoro-uracil, the nucleotide will be converted to 5-fluoro-deoxyuridine monophosphate, FdUMP. The natural development of dUMP is to form deoxythymidin monophosphate, dTMP, supplied with a methylen-group, CH_2, from methylentetrahydrofolate. This process is enzymatically driven by thymidylate synthase, TS. dTMP is further transformed to deoxythymidindiphosphate, dTDP, and deoxythymidintriphosphate, dTTP, by pyrimidine monophosphate kinase and pyrimidine diphosphate kinase, respectively. The nucleotide dTTP is incorporated into the DNA molecule, formed by several nucleotides which are linked together to form a single strand of DNA.

FdUMP will not develop further the way dUMP develops to form dTMP. Methylentetrahydrofolate, instead of giving a methylen-group, will form a stable complexe with TS and FdUMP. The phosphorylation by pyrimidine monophosphate kinase and pyrimidine diphosphate kinase will form FdUDP and FdUTP. FdUTP will subsequently be incorporated into the DNA molecule instead of dTTP. A damaged DNA is then formed by a DNA single strand interruption, a so-called nick (Grem 1990, Mathews and van Holde 1996).

A DNA single strand interruption will be discovered in the G_1 cell cycle checkpoint, which will trigger an apoptotic cell death. The aim of causing cell damage, that will lead to cell death, has thus been accomplished.

2. Incorporation into RNA and growth inhibition

5-FU is also incorporated in uridinmonophosphate, UMP, to form FUMP. FUMP is phosphorylated to FUDP and FUTP. FUTP is incorporated instead of UTP in RNA. Incorporation of FUTP into RNA may profoundly alter RNA processing and function (Glazer and Peale 1979) although the specific molecular locus for cytotoxicity by an RNA-related mechanism remains to be clarified. RNA synthesis as well as protein synthesis will be affected both quantitatively and qualitatively (Frösing 1994).

Catabolic pathway

The catabolic route for 5-FU is by enzymatic degradation. 5-FU is extensively catabolised in the liver through the rate limiting enzyme dihydropyrimidine dehydrogenase (DPD). DPD is also expressed in various other normal tissues, such as the gastrointestinal mucosa, as well as in tumour tissues. Several studies have demonstrated the clinical importance of DPD levels in cancer patients, suggesting that the efficacy of 5-FU may be related to DPD activity in tumour tissue. Indeed, DPD activity may be useful in determining tumour cell sensitivity to 5-FU. The lower the DPD activity, the greater the 5-FU efficacy. Patients with a DPD deficiency may exhibit severe 5-FU-related toxicities (Inada *et al.* 2000; Milano and Etienne 1994).

Adverse effects of 5-FU

As pointed out, an obstacle with cancer chemotherapy is the effects on normally proliferating tissues. Consistently, the main toxicities of 5-FU are exerted on rapidly dividing tissues, primarily the oropharyngeal and gastrointestinal mucosa and bone marrow. 5-FU toxicity varies with dose, schedule and route of administration. Considerable variation in the incidence and severity is observed among patients. Diarrhoea, mucositis, myelosuppression, nausea and alopecia are the most frequently reported side effects (Curreri and Ansfield 1962)

CELL DEATH

Since the ultimate goal of 5-FU treatment is to cause cell injury, leading to cell death in tumour cells, it is of value when studying adverse effects of this drug, to clarify the final fate of cells after the induction of DNA strand breaks. To put cell death by cancer chemotherapy into its proper context, the main types of cell deaths will be reviewed.

Apoptosis

Normally, in proliferating tissues, constant cell renewal by mitosis is balanced by programmed cell death in the maintenance of tissue homeostasis (Wyllie *et al.* 1980). Programmed cell death is an induced intrinsic and orderly cell death process, which presents itself with distinct morphologic changes, referred to as *apoptosis*.

Although the phenomenon of programmed cell death was observed already in the 19th century, (for review see, Majno and Joris 1995) it was first described in detail and named apoptosis by Kerr *et al.* in 1972 (Kerr *et al.* 1972). The process involves the breakdown of mitochondrial membrane potential and release of cytochrome C and apoptosis inducing factor (AIF) (Hortelano *et al.* 1997), which in turn can activate special proteases, the caspases. Bcl-2 proteins on the other hand may inhibit apoptosis. Caspases are present in the cytoplasm as inactive proenzymes that require proteolytic cleavage to become activated. Nucleases, which are proteolytic enzymes that cleave nucleic acid, will eventually degrade chromosomal DNA. This leads to multiple nicks and strand breaks within the DNA molecules and results in the generation of DNA oligomers of the size proportional to the size of a nucleosome, approximately 180 bp (Mathews and van Holde 1996). A systematic and orderly disassembly of the cell has started. When scattered cells die by apotosis, no inflammatory reaction is elicited, since the cell remnants, i.e. apoptotic bodies, are phagocytosed by neighbouring epithelial cells or macrophages, before plasma membrane integrity is lost (for review see, Savill *et al.* 1993).

Although apoptosis is a normal physiologic process balancing mitosis, it can also be triggered in a cell by a distorted gene, as an intrinsic suicide process to dispose of cells with potentially dangerous gene mutations. A distorted gene, in a potentially malignant cell or a virus infected cell, is discovered at cell cycle checkpoints during proliferation. These checkpoints reveal the distorted DNA molecule and, if the DNA cannot be repaired, the intrinsic cell death program is induced via the protein p53 and caspase activation. Some malignant cells may obtain the ability to escape the intrinsic p53 checkpoint system, or distort it so that the cancer cells can survive.

Cytotoxic insults, e.g. radiotherapy or cancer chemotherapy may also kill cells by apoptosis. When treating with an anticancer drug, like 5-FU, the drug is built into proliferating cells. A new gene damage is created within the proliferating cell, and the cell may go into apoptosis, activating intrinsic death mechanisms. That way the drug will assist the body with disposing of malignant cells. Since tumour cells often have a high turnover rate, anticancer drugs will target these cells.

Furthermore, apoptosis can be induced in cells by external signals from other cells within the body. Cytotoxic T lymphocytes (CTL) can bind to a protein on the cell membrane on the target cell or release cytotoxic proteins, which enter the target cell, and in turn induce apoptosis. Also bacterial lipopolysaccarides (LPS) may induce apoptosis in target cells (Besnard *et al.* 2001; Kawahara *et al.* 2001). A cascade of caspases is activated within the target cell, which drives the apoptotic process.

Necrosis

In contrast to apoptosis, *necrosis* is a passive type of cell death without regulatory mechanisms, a catastrophic event caused by major insults. When necrosis is induced, it usually occurs within tracts of contiguous cells. Cells lose their plasma membrane integrity at an early phase in the process. Necrosis includes cell swelling and bursting, disintegration of the cell membrane and release of intracellular debris within the tissue, which triggers phagocytosis, inflammation and tissue damage (Denecker *et al.* 2001).

Autophagic degeneration

An additional form of cell death has also been recognised, *type 2 cell death* or *autophagic degeneration* (Clarke 1990), described as a type of programmed cell death with partly other features than apoptosis. It is characterised primarily by the formation of numerous autophagic vacuoles in the cytoplasm. The nucleus is normal or shows chromatin condensation centrally in the nucleus. Epithelial cells may lose junctional complexes. Blebbing, as in apoptosis, may occur but also endocytosis, or "inward blebbing" which serves well to reduce the area of the plasma membrane. Although autophagic degeneration has been suggested to be a programmed form of cell death, it still has some features in common with necrosis. For example it has been suggested that the uptake and removal of cell remnants involve inflammation (Kitanaka and Kuchino 1999).

ORAL EPITHELIUM

Epithelial tissues line all cavities and free surfaces of the body. All epithelia have at least one important function in common, they serve as selective permeability barriers, separating fluids on each side that have different chemical compositions (Alberts *et al.* 1994). The integrity of the epithelium also acts as a physical barrier to prevent penetration of micro-organisms (Marsh and Martin 1999; Alberts *et al.* 1994). An undamaged epithelium is the first line of defence, and may be crucial for the maintenance of body homeostasis.

Normal structure

The oral cavity is lined by a *stratified squamous epithelium*. This type of epithelium is also present in the epidermis, the pharynx, eosophagus and a number of other body surfaces. In humans, the mucosa of the oral cavity is non-keratinised as well as keratinised. The latter is found on oral mucosal surfaces most exposed to masticatory forces, i.e. the hard palate, gingiva and tongue. In rodents the buccal epithelium and epithelium of other oral lining mucosa is orthokeratinised (Chen SY and Squier CA 1984; Squier and Kremer 2001).

The oral epithelium, like other epithelial tissues, is composed mainly of *keratinocytes*, *matrices* and *junctions* between these constituents. Some other cells, i.e. melanocytes and immunocompetent cells, also reside within the oral epithelium.

Oral keratinocytes

The keratinocytes are renewed by proliferation of immortal stem cells in the basal cell layer. The cells differentiate to mature keratinocytes higher up in the epithelium. The renewal rate of buccal mucosal epithelium has been estimated to 10-14 days in humans (Chen SY and Squier CA 1984) and rats (Karring and Loe 1972), which is slower than the single layer epithelium of the intestinal columnar epithelium, but faster than the keratinised epidermal stratified squamous epithelium of the skin.

The oral keratinocytes were earlier thought to exert mainly passive barrier functions between the external environment and subepithelial tissues. Research in recent years has shown that the epithelial cells are actively involved in recognition of antigens, e.g. bacterial toxins, and signalling to underlying tissues as well as receiving signals from these tissues. Several

cytokines are involved in this process (Lourbakos *et al.* 2001; Miyauchi *et al.* 2001; Sandros *et al.* 2000). The cells also show selective permeability (Sloan *et al.* 1991). Gingival keratinocytes may be invaded by whole bacteria (Madianos *et al.* 1996). It cannot be ruled out that keratinocytes in other parts of the oral cavity, may also be invaded by bacteria.

Matrices of the oral epithelium

The intercellular substance, the matrix, is relatively sparse in the epithelium, compared to many other tissues. It plays a role in adhesion but also acts a lubricant, facilitating the sliding of cells past one another and as a medium that can regulate the diffusion of substances through the intercellular channels of the epithelium. The water-containing intercellular matrix can facilitate diffusion and selective binding of metabolites (Gerson SJ and Harris RR 1984).

Basal lamina

The basal lamina underlies all epithelial cell sheets, separating epithelial cells from underlying connective tissues. It is composed of extracellular matrix materials including protein type IV collagen, which is synthesised by the epithelial cells. The basal lamina influences many functions, e.g. cell metabolism, differentiation and migrations. Substances moving from the connective tissue to the epithelium and vice versa must pass the basal lamina. Indeed, the basal lamina may serve as a rate-limiting barrier to some substances and endotoxins (Alfano *et al.* 1977; Alfano *et al.* 1975) and is thus important in the context of epithelial barrier functions.

Cell junctions

Epithelial cell adhesion molecules are involved in the adhesion between cells and between cell and matrix. Intact adhesion mechanisms are of crucial importance for the maintenance of epithelial barrier functions. Impairment may be detrimental for mucosal homeostasis. The cell junctions are of three kinds, anchoring junctions which mechanically attach cells (adherence junctions, desmosomes, hemidesmosomes), occluding junctions which seal cells together (tight junctions) and communicating junctions which mediate signals between adjacent cells.
Some function of sealing both keratinised and nonkeratinised epithelial cells in oral epithelium may be done by intercellular material, derived from vesicles, the membrane-coating granules of stratum granulosum (Chen and Squier 1984).

5-FU and oral epithelium

In early studies it was noted that topical administration of 5-FU to skin created discontinuities in the basal lamina and widened intercellular spaces. Cell tonofilaments were reduced and condensed, and mitochondrial degeneration and irregularities in nucleoli were seen. Many degenerating keratinocytes detached (Hodge *et al.* 1975). Electron microscopy demonstrated cytoplasmic vacuoles and alterations in the mitochondria in the keratinocytes (Zelickson *et al.* 1975). Other studies demonstrated that epidermal growth factor, a molecule known to stimulate epidermal cell division, increased oral mucosal breakdown during 5-FU therapy. The hypothesis was presented that the epithelial basal cell proliferation rate is one of the key elements in determining mucosal sensitivity to cancer chemotherapy (Sonis *et al.* 1992). Accordingly, the transforming growth factor-beta (TGF-beta) family of regulatory growth factors, which reversibly arrest cell division in the G_1 phase of the cell cycle was shown to protect epithelial cells from cytotoxic damage by 5-FU in animal studies. TGF-beta also reduced the severity and duration of oral mucositis in animal studies (McCormack *et al.* 1997; Sonis *et al.* 1994). However, in a recent study, no advantage of TGF-beta3 treatment regarding the incidence of oral mucositis was seen in a group of patients on cancer chemotherapy (Foncuberta *et al.* 2001).

After 5-FU treatment, large molecules have been shown to pass through the epithelium, implying that 5-FU may alter the epithelial barrier function. Interestingly, the number of low differentiated viable buccal cells, obtained by oral washings, increased after high-dose chemotherapy with 5-FU, implying that cells were easily detached (Wymenga *et al.* 1997). Some studies in recent years also involve cytokines in the search for effective treatments against mucositis (Sonis *et al.* 2000).

It seems clear from these studies, that the oral epithelium is affected by 5-FU treatment. Since the oral epithelium is an important barrier function, cell death by 5-FU in the oral keratinocytes was studied in this thesis.

Cellular immune defence

Normal structure

The immune system protects the body from infection, foreign molecules and cancer, thereby constituting a major barrier function. The immune system has both non-specific and specific components, together making up the innate and adaptive immunity. The two systems operate in concert to defend the host.

The cellular compartment of the immune defence comprises three main categories of bone marrow derived cells, the granulocytes (neutrophils, eosinophils, basophils and mast cells), the monocytes (macrophages and dendritic cells) and lymphocytes (T and B lymphocytes and natural killer (NK) cells) (Alberts *et al.* 1994).

Cells of the innate immune system of the oral mucosa

The innate immunity often provides the first reaction after an antigen challenge (Goldsby 2000). Granulocytes, macrophages and NK cells are the major components, neutrophils and macrophages being the main professional phagocytes in the body. In the oral mucosa the neutrophils are rarely encountered in healthy oral mucosa. They circulate for only a few hours in the blood stream and survive for a couple of days after entering an insulted tissue. Macrophages are normally found in the mucosa and survive for a long time, even months, in the tissues. The macrophages are located in the lamina propria of the oral mucosa.
In a study on inflammatory response, no significant numbers of NK cells were found in oral mucosa of rats (Matthews *et al.* 1986).

Tissue mast cells are located in the deep lamina propria of oral mucosa, around blood vessels and salivary secretory ducts, in close association with nerve endings and between muscle fibers (Fortier *et al.* 1990). Mast cells may possibly have a lifespan as long as that of the animal itself, and may be able to regenerate granules after degranulation (Padawer 1974).

Cells of the adaptive immune system in oral mucosa

Dendritic cells (DC), called Langerhans cells (LC) in the ectodermal tissue of both oral mucosa and skin, T lymphocytes and B lymphocytes are all cells of the adaptive immune response. DC cells are found mainly in stratum basale of the epithelium and in the lamina

propria of the connective tissue. The half-life of DC has been reported to be somewhat different in different tissues, 2 days in airways, 7 days in lung parenchyma and 9 days in epidermis in mice (Ghaznawie *et al.* 1999b). There are also reports on a longer half-life, up to several months in human epidermis.

The lymphocytes, which originate from the lymphoid stem cells, recycle between the lymph nodes, blood, tissues and lymph vessels and are found in large numbers in the lymph nodes. In the oral mucosa scattered T lymphocytes are found in the epithelium, otherwise mainly in the lamina propria, more prominently in the layer adjacent to the epithelium than in deeper layers (Lebendiger and Lehner 1981; Lundqvist and Hammarstrom 1993). B cells are seldom found in normal healthy oral mucosa.

Cellular immunity of the dental pulp

The dental pulp is a connective tissue structure enclosed and protected by rigid mineralised tissues with little exposure to bacterial challenges while the tooth structure remains intact. In the normal healthy dental pulp, subsets of both macrophages and dendritic MHC class II molecule expressing cells have been identified (Jontell *et al.* 1988; Okiji *et al.* 1992a; Okiji *et al.* 1992b). DC reside mainly in the periphery of the pulpal connective tissue but are also found scattered within the central portion of the pulp (Jontell *et al.* 1987). Studies indicate that macrophages on the other hand are evenly distributed within the pulp and that macrophages outnumber DC (Okiji 1992). In experimental pulpitis, MHC class II molecule expressing macrophages and DC rapidly increase following LPS challenge (Bergenholtz *et al.* 1991). A small number of T lymphocytes have also been observed. They represent both $CD4^{+}$ T_H cells and $CD8^{+}$ CTL (Jontell *et al.* 1987; Hahn *et al.* 1989). The presence of B lymphocytes is controversial. According to Okiji it seems difficult to justify a significant role of B lymphocytes in the normal dental pulp, as it has only rarely been identified in this tissue (Hahn *et al.* 1989).

Immune function of the cellular adaptive immune system

Foreign antigens that are taken up by DC, are processed within the cell. An antigen that has been internalised is degraded into oligopeptides by an endocytic pathway. The peptides bind to class II molecules, derived from major histocompatibility complex (MHC) within the cell. When a peptid has bound to MHC class II, the complex is transported to the plasma membrane and expressed on the cell surface. Only professional antigen presenting cells, i.e.

DC and macrophages of the cellular immune defence (and the B lymphocytes of the humoral immune defence), are able to present antigens together with MHC class II molecules and also deliver the co-stimulatory signal necessary to activate $CD4^+$ helper T lymphocytes (T_H). After internalising foreign antigen, the DC migrate to regional lymph nodes for antigen presentation (Silberberg *et al.* 1989). During the migratory process they upregulate their synthesis of MHC class II molecules. The interaction between the processed antigenic peptide, bound to a MHC class II molecule on the surface of the dendritic cell, and a T_H cell receptor (TCR)-CD3 complex on the surface of the T_H cells, together with the second, co-stimulatory, signal and other membrane molecules, activate the T_H. This will start a cascade of biochemical events. Activation leads to proliferation and differentiation and further stimulation of immunocompetent cells to exert barrier functions of the immune defence.

In contrast to exogenous antigen, processed and presented by an endocytic processing pathway and presented together with MHC class II, endogenous antigens are degraded within the cytoplasm into peptides that can bind to MHC class I molecules. Virtually all cells in the body express MHC class I. CTL can bind via its T cell receptor (TCR) to MHC class I molecules that are presenting an antigen, and induce apoptosis.

Lymph nodes

Lymph nodes are encapsulated structures specialised for trapping antigen from local tissues. The lymph nodes contain a network, packed with T and B lymphocytes, macrophages and DC. Antigens may be carried into the regional lymph nodes by the lymph. An antigen that is brought in with the lymph, will be trapped by a cellular network of phagocytic cells and dendritic cells. Other antigens will be captured out in the peripheral tissues of the body at the site of entrance by DC, be processed by the DC, which will in turn migrate through the lymphatics to the regional lymph nodes.

The cervical lymph nodes, used throughout the works of this thesis, were the mandibular lymph nodes of the rat. These lymph nodes drain the oral cavity, tongue included, salivary glands, external ear and rostral head region (Komarék V. *et al.* 2000). The mesenteric lymph nodes, which were used in these studies, drain the distal duodenum, ileum, cecum and proximal colon (Komarék V. *et al.* 2000; Gautreaux *et al.* 1994).

5-FU and the immune defence

5-FU exerts toxic effects on bone marrow cells, causing myelosuppression and granulocyopenia as an adverse effect to treatment (Harrison *et al.* 1978; Vetvicka *et al.* 1990). The 5-FU effects on cells of the immune system are also illustrated by the loss in spleen weight in 5-FU treated animals (Carlsson *et al.* 1995; Ohta *et al.* 1980). 5-FU has also been shown to negatively influence the antigen-presenting capacity of spleen cells in mice (Nakano *et al.* 1991). Studies on 5-FU effects on macrophages have shown that the drug appears to inhibit immature macrophage, but not mature effector cells (Athlin and Domellof 1987; Connolly *et al.* 1983; Vetvicka *et al.* 1990). In an early study B and T cell lines both showed sensitivity to 5-FU, B cells more than T cells (Ohnuma *et al.* 1978).

Thus, 5-FU influences several immunocompetent cells. Oral mucositis, induced by 5-FU, most likely at some stage involves immunocompetent cells and cytokines. Yet, current knowledge is incomplete on the effects of 5-FU on immunocompetent cells of the oral mucosa. Since immunocompetent cells of the oral mucosa exert major barrier functions against tissue damage and invading pathogens, knowledge is warranted on how these cells are affected.

MICROFLORA

Body homeostasis requires an internal environment free from bacteria and a balanced microflora in organs in direct contact with the external environment, e.g. the alimentary tract. The ability of the microflora to maintain stability in the oropharyngeal and gastrointestinal tract constitutes an important barrier function.

Normal conditions in the oral cavity and intestine

Bacteria

The oral cavity, with its moist environment, rich in nutrients from ingested food and salivary components, harbours an abundant indigenous microflora. There are four major ecosystems in the mouth, the buccal mucosa, the dorsum of the tongue, the supragingival tooth surface and

the crevice area (Schonfeld 1992). Also the intestine harbours an abundant indigenous flora, where the colon is the site, most rich in micro-organisms with 400-500 species (Berg 1996).

Oral bacteria are mainly facultative anaerobes, both in humans and rats. Buccal mucosa and other oral lining mucosa harbour mostly gram-positive cocci, mainly *Streptococcus* spp (Schonfeld 1992). Lactobacilli are sparse in healthy humans (Marsh and Martin 1999). The tongue, with its papillary surface, harbours more bacteria per epithelial cell than other oral mucosal surfaces (Marsh and Martin 1999). Also on the tongue *Streptococcus* spp dominate the flora, however less pronounced, compared to the buccal mucosa. Some obligate anaerobes are found on the tongue.

In the upper small intestine the main types of bacteria are acid-tolerant lactobacilli and streptococci. The large intestine is inhabited mainly by obligate anaerobes, 100-1000-fold more numerous than facultative anaerobes (Berg 1996).

Bacterial translocation

Bacterial translocation has been defined by Berg and Garlington 1979 (Berg and Garlington 1979) as the passage of viable bacteria from the gastrointestinal tract through the epithelial mucosa into the lamina propria and to the mesenteric lymph nodes and other tissues. There have been suggestions to extend the definition to nonviable bacteria and microbial products such as endotoxins (Alexander 1990).

A low degree of translocation may possibly occur under normal conditions. The presence of indigenous translocating bacteria in low numbers to the lamina propria and mesenteric lymph nodes has been suggested to be a normal beneficial mechanism for stimulating the host immune system to respond more quickly to exogenous pathogens (Berg 1996).

Translocation has been shown to increase by physical disruption of the intestinal epithelium, compromised immune system and bacterial overgrowth (Berg *et al.* 1988). Enterobacteria like *E. coli* and enterococci have a transmucosal route of translocation (Alexander *et al.* 1990) and bacteremia caused by gram-negative bacteria from the gastrointestinal tract may occur (Kreger *et al.* 1980a; Kreger *et al.* 1980b). Some studies have examined how microbes pass through the intestinal epithelial barrier. In an experimental study of burn injuries, *Candida* spp. and *E. coli* passed through the enterocytes in the ileum and not between the cells, to the

lamina propria (Alexander *et al.* 1990). Some bacteria seem to translocate easier than others. Hence, Berg found that indigenous gram-negative enteric bacteria translocated in large numbers, gram-positive bacteria at intermediate levels and obligate anaerobes translocated at only very low levels (Steffen *et al.* 1988).

Translocation of bacteria has been shown to occur with similar intensity throughout the gut, however more bacteria were shown to be killed in the process of translocation across the lower part of the intestinal tract (Fukushima *et al.* 1994). The path for bacteria to the different parts of the mesenteric lymph nodes can be related to their population levels in the regions of the gastrointestinal tract, that provide lymph to the various lymph node segments (Gautreaux *et al.* 1994). *E. coli,* for example, was most frequent in ascending colon, cecum and distal ileum, less frequent in proximal ileum and least frequent in jejunum (Steffen and Berg 1983).

The oral cavity as a source for bacterial dissemination

The oral cavity has for many years been considered as a source for bacterial dissemination. Bacteria from the oral cavity have been identified in the blood (bacteremia), e.g. in patients after dental procedures (Daly *et al.* 2001; Hall *et al.* 1996) and in bone marrow transplanted patients treated with methotrexate (Heimdahl *et al.* 1989).

The proposed theory in this thesis of the cervical lymph nodes as a bacterial dissemination pathway after 5-FU treatment is supported by knowledge on lymph drainage. Furthermore, in a recent clinical study bacteria were found in neck regional lymph nodes from patients with oral carcinoma (Sakamoto *et al.* 1999).

Probiotic bacteria

A probiotic substance is a live microbial feed supplement, which affects the host animal by improving its intestinal microbial balance (Fuller and Berg 1985). Lactic acid producing bacteria, such as lactobacilli, have such properties. Some studies have shown, that bacterial overgrowth in the intestine, and translocation from the gastrointestinal tract to the mesenteric lymph nodes, could be hampered by supplying the gastrointestinal tract with probiotic bacteria. These bacteria may suppress the growth of potential pathogens. *Lactobacillus plantarum* constitutes a large part of *Lactobacillus* spp of human gut (Ahrne *et al.* 1998).

The strain *L. plantarum* 299v was originally isolated from human intestine (Molin *et al.* 1993). The strain has been shown to have the capacity to colonise rat intestine (Herias *et al.* 1999). It has probiotic properties. Fed to rats treated with the anticancer drug methotrexate, it decreased translocation initiated by methotrexate treatment (Mao *et al.* 1996).

5-FU and the microflora

5-FU treatment may disturb the gastrointestinal microflora (Nomoto and Yokokura 1992). Microbial distortions in the intestine in connection with 5-FU treatment have been shown to cause bacterial spread to mesenteric lymph nodes and disseminated infections (Deng *et al.* 1999; Nomoto *et al.* 1991; Sandovsky-Losica *et al.* 1992). Nonspecific immunostimulation augments host resistance against the lethal toxicity of 5-FU in tumour-bearing mice by intravenous administration of a preparation of heat-killed *Lactobacillus casei* (Nomoto and Yokokura 1992).

Thorough knowledge of bacterial disturbances by 5-FU in the oral cavity is lacking. There seems however to be a bacterial component in oral mucositis during 5-FU treatment, as shown by a decreased mucositis in 5-FU treated hamsters, that received antibacterial treatment (Loury *et al.* 1999). Despite an intensive discussion on the oral cavity as a source for bacteremia and infections, the cervical lymph nodes as a dissemination pathway for bacteria from the oral cavity has not received adequate attention.

Material and Methods

The protocols for animal experiments carried out within the framework of this thesis were approved by the Animal Ethics Committee in Göteborg, Sweden or by the Otago University Animal Ethics Committee in Dunedin, New Zealand.

Animals (I-IV)

Inbred female Lewis rats, 175-200 g, were used for the functional studies on T cell proliferation (III), since cells from several animals have to be pooled to reach a satisfactory number for each experiment. This requires cells from immunologically identical animals. For reasons of uniformity, the same breed was used throughout the works of this thesis. Before and after injection periods each animal was consistantly weighed.

5-Fluorouracil injections (I-IV)

The rats were given bolus injections of 5-FU in the tail vein. Injections were given at 30 mg/kg (II, III) or 50 mg/kg (I-IV) for one or three consecutive days (I) or on days 0, 1, 2, 5, 6 and 7 (II-IV). The animals were killed at different time intervals after the last injection by carbon dioxide inhalation followed by cervical dislocation.

Probiotic bacterial treatment (IV)

L. plantarum 299v was tested for resistance to 5-FU. Ten µl of 5-FU 50 mg/ml was applied to paper discs on blood agar plates spread with *L. plantarum* 299v and incubated aerobically for 48 hours. *L. plantarum* 299v showed resistance to 5-FU and was therefore added to the drinking water to the animals in one experiment, to investigate its capacity to restrain bacterial overgrowth during treatment with 5-FU.

For this purpose *L. plantarum* 299v was cultured aerobically on Rogosa agar medium at 37°C for 48 h, harvested in tap water and adjusted to a concentration of 10^9/ml as determined by optical density. In one experiment fresh bacterial suspensions were administered to the rats each day during the experimental period, starting three days before 5-FU injections. *L. plantarum* express a mannose-specific adhesin, which mediates binding to intestinal epithelial cells and contributes to the colonising capacity of the bacteria. This adhesin also agglutinates to yeast cells. The *L. plantarum* 299v cultures were tested each day for their ability to

agglutinate the yeast *Saccharomyces cerevisiae*. *L. plantarum* 299v mutated strain, not expressing the adhesin, was used as a negative control.

Tissue sampling (I-IV)

After the animals were killed the following tissues were collected.

Blood samples were collected after cardiac puncture and transferred to EDTA-containing vials (II).

Buccal mucosa was dissected and freed from muscle layers (I-IV)

Cervical (submandibular) and mesenteric lymph nodes were carefully dissected aseptically (III-IV)

Spleens were extirpated in one experiment, and the wet weights of the spleens were registered (unpublished data).

Tongue samples were taken by cutting the tip of the tongue, 5 mm of lenght. The method was evaluated by weighing the pieces. The method proved satisfactory reproducibility (IV).

Dental pulps were removed with a fine forcepts from maxillary incisors that had been carefully extracted and split open (II, III).

Intestinal content (7 μl) was collected half way between the lower gastric sphincter and the cecum and from the proximal part of the colon, by calibrated stainless steel spoons (Jordan *et al.* 1968) (IV).

Blood cell counts (II)

Blood samples for determination of leukocyte count, differential counts, haemoglobin and platelet count, were collected by cardiac puncture immediately after the animals were killed. Determination was performed to evaluate the influence of the 5-FU dosages on bone marrow derived cells in blood circulation.

High-performance liquid chromatography (HPLC) for determination of 5-FU plasma levels

Quantitative determination to establish 5-FU plasma levels in 30 mg/kg (n=5) and 50 mg/kg (n=5) 5-FU injected animals was performed. Two animals were used as controls. The animals were sacrificed in conjunction with the last injection and the thorax was cut open. Cardiac blood samples were taken 5 min after the 5-FU injection and transferred to heparin-containing vials. 5-FU plasma concentrations were determined by HPLC (unpublished data).

Diarrhoea (IV)

Diarrhoea has been defined as an increase in frequency of stools and in water content of the stool (Jeejeebhoy 1977). The water content of the intestinal lumenal content was estimated as an indicator of the likelihood of diarrhoea. 15 µl of intestinal content was placed on a filter paper and the diameter of the wet zone created around the probe was measured in a blinded way and was used as the measurement.

Cell death detection (I)

From untreated controls and 5-FU treated animals paraffin- or resin embedded buccal epithelium and buccal epithelial cellsuspensions were analysed according to the methods described in Table 1.

Table 1. Methods used for cell death analyses

Analysis	Method	Description	Aim	Source
(i) Cell sorting	Flow cytometry (fluorescence-activated cell sorting-FACS)	Analysis of the light signals from particles flowing in a stream past a focused light beam	Discriminate between cells according to cell size and granularity (Dive *et al.* 1992)	FACScan; Becton-Dickinson
(ii) Cell staining	Vital dye (Trypan Blue) exclusion test and cell counting using a light microscope	Discrimination of viable (intact plasma membrane) and dead (damaged plasma membrane) cells. Cells with disturbed plasma membrane permeability stain blue, undamaged (viable) cells appear translucent	Discriminate between live and dead cells	
(iii) Immunohisto-chemistry	Terminal deoxynucleotidyl transferase (TdT) mediated dUTP-biotin nick en labeling (TUNEL) method with peroxidase staining	Nucleotide that binds to DNA strand breaks was added to sections of paraffin-embedded buccal mucosa. Adjustments of the manufacturer protocol was done to overcome problems of overstaining	Detect and estimate number of cells with apoptotic DNA fragmentation (Gavrieli *et al.* 1992)	Boehringer Mannheim
(iv) Ultrastructural analysis	Transmission electron microscopy (TEM)	Analysis of cell and tissue morphology in ultrathin sections (70 nm)	Visualise structural changes in the cells (Kerr 1972)	Zeiss CEM 902 electron microscope

Estimation of cell numbers by stereology (I)

A stereological technique (Gundersen 1986) for quantifying biological structures was used to estimate the number of nuclei with DNA strand breaks and the total number of nuclei, i.e. the number of cells, per volume of tissue in the basal cell layer of paraffin embedded buccal epithelium. Five pairs of adjacent sections, randomly selected, were examined using a light microscope with an attached colour video camera connected to a computer. A systematic random sampling technique was applied to select the same field under the microscope in both sections of each pair. The images were saved onto the computer, printed and used for counting. The disector principle (Gundersen 1986) was applied for counting. Three counting frames of the same size were placed in a standardised manner on each image, covering areas of basal cell layer. The same areas were covered in both images of a pair. Only nuclei that were not intersected in both sections of a pair were counted. The mean number of stained cells and the mean total number of cells per volume of tissue was calculated as described in paper I, giving the mean number per volume of basal cell layer.

Identification of immunocompetent cells by immunohistochemistry (II)

DC, macrophages and T lymphocytes were identified by cell surface markers. Immunohistochemical studies, using monoclonal antibodies against the cell surface markers, were performed on frozen tissue sections of buccal mucosa and dental pulp. *LC* of the buccal epithelium and *DC* of the lamina propria and dental pulp were identified by a mouse monoclonal IgG anti-rat MHC class II (OX-6). *Macrophages* were identified by mouse monoclonal IgG anti-rat ED2-like molecule (HIS36). HIS36 reacts with an ED2-like antigen, which is found on tissue and exudate macrophages (Yamin *et al.* 1990). *T lymphocytes* were labeled using a mouse monoclonal IgG anti-rat CD2 that binds to CD2 molecules on peripheral T cells. CD2 is expressed on both CTL and T_H as well as on NK cells. A secondary antibody and peroxidas staining was used to visualise the cells using a light microscope. The stained cells were counted on projected images (epithelium) or using an ocular grid to define consecutive fields of the sections.

The *LC* of the buccal epithelium were also visualised and counted in epithelial sheets, after labelling of MHC class II molecule expressing cells with fluoroscein isothyocyanate (FITC)-conjugated OX-6. A light microscope with a fluorescent illuminator was used and photos of three cell-rich areas from each sheet were taken, blinded and analysed for numbers of stained cells.

Identification of MHC class II expressing cells by flow cytometry (FACS) (II)

The MHC class II expressing cells of the buccal epithelium were also identified by flow cytometry. Epithelial cell suspensions were prepared as described in paper II. The cell suspensions were incubated with FITC-conjugated OX-6. MHC class II molecule expressing cells were identified and counted by flow cytometry (FACSCan; Becton Dickinson).

Immunofunctional assay (III)

For immunofunctional assays, suspensions of pooled buccal epithelial cells or pulpal cells, including MHC class II molecule expressing cells, were prepared from up to 15 rats for each suspension. Suspensions from untreated control animals and 5-FU treated animals were prepared. Suspensions of T lymphocytes were prepared from lymph nodes of untreated rats. The T lymphocytes were extracted by negative selection, using mouse monoclonal IgG anti-rat MHC class II molecule (OX-6) and goat anti-mouse IgG conjugated immunomagnetic beeds to remove MHC class II molecule expressing cells, the macrophages, DC and B-lymphocytes. An additional technique using nylon wool columns to remove B-lymphocytes was used in one experiment. The epithelial cell suspensions from untreated or 5-FU treated animals were incubated together with T cell suspensions from lymph nodes of untreated animals. Concanavalin A (ConA) was added to stimulate T cell proliferation. [^{3}H]-thymidine was added following 48 h of incubation. The capacity of MHC class II molecule expressing cells of the epithelial or pulpal cell suspensions to provide accessory signals for T cell proliferation was evaluated by measuring [^{3}H]-thymidine uptake in T cells after an additional 24 h, as a measure of proliferation.

The T cell suspensions were checked for purity from MHC class II molecule expressing cells on day 1 and 3 by adding FITC conjugated OX-6 to the suspensions and analysing by flow cytometry. The purification of the T cell suspensions was also checked by adding ConA and [^{3}H]-thymidine, but no MHC class II molecule expressing cells, and measuring T cell uptake of [^{3}H]-thymidine. The values are expected to be very low in both cases.

In a complementary *in vitro* study, $CD4^{+}$ and $CD8^{+}$ T lymphocytes from lymph nodes were analysed for sensitivity to 5-FU (unpublished data). Suspensions of T lymphocytes from lymph nodes of untreated rats were prepared. 5-FU in a concentration of 150 µg/ml was added and the cellsuspensions were incubated for 12 h in 37°C. T cell suspensions without 5-FU

were incubated as controls. A Ficoll-Paque density gradient centrifugation was performed to remove dead cell. The cell suspensions were incubated with a mouse monoclonal IgG anti-rat CD4 (clone W3/25; Serotec, Oxford, UK) or a mouse monoclonal IgG anti-rat CD8 (clone MRC OX-8; Serotec, Oxford, UK). A phycoerythrin(RPE)-conjugated secondary rabbit monoclonal IgG anti-mouse (DAKO A/S, Glostrup, Denmark) was added and flow cytometric analysis was performed. The proportions of $CD4^+$ and $CD8^+$ cells in the cell suspensions were estimated.

Bacterial levels in the oropharyngeal and gastrointestinal tract and lymph nodes (IV)

Oral biopsies (tongue, buccal mucosa), samples of small intestinal and colonic lumenal content and lymph node homogenates were incubated for 2 days aerobically and for 5 days in 95% hydrogen and 5% carbon dioxide on blood agar plates. Rogosa agar culturing was done for 2-3 days in 90% nitrogen and 10% carbon dioxide, for identification of *Lactobacillus* spp. Colonies with different morphological appearances from aerobically incubated blood agar plates and Rogosa agar were grouped according to gram-staining appearance. Lymph node homogenates were also cultured aerobically overnight on Drigalski agar for the identification of enterobacteria.

Statistical analyses (I-IV)

Parametric and non-parametric methods were used for statistical analyses using the computorised StatView program (SAS Institute Inc. North Carolina). Percentages and log data were evaluated using Student´s *t* test for unpaired data (II, IV) or matched paired data (III). Statistical tests for differences in the number of keratinocytes between groups were performed according to Kruskal-Wallis and Mann-Whitney *U* tests (I). Bacterial translocation data were analysed by Mann-Whitney *U* test (IV). Kappa statistics was used to test reliability of the scoring when estimation of cell number by stereology was performed (I).

RESULTS AND DISCUSSION

THE ANIMAL MODEL (I-IV)

5-FU serum concentrations in the Lewis rats after the doses used in these studies (30 mg/kg and 50 mg/kg *i.v.* bolus injections) are shown in Fig. 1.

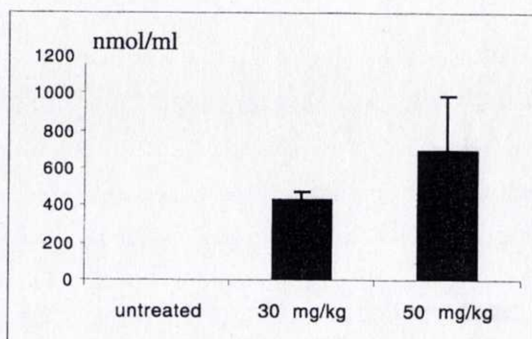

Fig. 1. Serum concentrations of 5-fluorouracil (5-FU), 5 min after bolus injection

WBC counts decreased significantly as expected after both low and high doses of 5-FU treatment, and virtually all granulocytes disappeared (II).

30 mg/kg 5-FU treated animals lost a mean of 5.2% of their *body weight* during the experimental period, $p<0.0001$. 50 mg/kg 5-FU treated animals had a body weight loss of 18.2% (S.D. 3.4), $p<0.0001$. The weight of the control animals slightly increased during the experimental period, a weight gain of 6.1% (2.9) was noted, $p<0.001$.

Spleen weights of untreated animals had a mean weight of 0.51 (0.03) g. In 30 mg/kg 5-FU treated rats, the spleen weights had decreased to 0.37 (0.04) g, $p<0.0001$, and in 50 mg/kg 5-FU animals to 0.24 (0.04) g, $p<0.001$.

The 5-FU serum concentrations in the rats were in the range of what can be found in humans after 5-FU treatment, given as a bolus injection. (Heggie *et al.* 1987; MacMillan *et al.* 1978).

The effects of 5-FU on WBC, rat body weights and spleen weights were in the same range as those registered in earlier 5-FU studies on rats that showed antitumour effects (Carlsson *et al.* 1995). It has been shown that after *i.v.* administration of 5-FU, the drug and metabolites are found in tumours, intestinal mucosa, bone marrow and liver in both humans and rats (Liss and Chadwick 1974). In summary, the data indicate that the rat model used throughout this thesis was adequate for the investigations.

ORAL EPITHELIAL BARRIER FUNCTION

Cell death in oral keratinocytes (I)

(i) Cells shrink and increase their granularity during programmed cell death, while during necrosis, cell swelling and loss of granularity occurs (for review see Cohen 1999; McConkey 1998).

The flow cytometry of oral epithelial cell suspensions revealed an increased number of small and granulated keratinocytes with increasing 5-FU doses, compared to cells from untreated animals (I, Fig 4.) (Tounekti *et al.* 1995).

These initial findings suggested that the oral keratinocytes had entered a programmed cell death pathway after the 5-FU treatment regimes, used in this study.

Exploring the theory of an apoptotic cell death in the keratinocytes further, gave the following results.

(ii) The cells kept their cell membrane integrity. This is a sign of apoptosis, since during apoptosis cells maintain their cell membrane integrity, while upon necrosis they lose cell membrane integrity.

(iii) The biochemical hallmark of apoptosis is degradation of the genomic DNA from activation of nuclear endonuclease. This enzyme cleaves DNA between nucleosomal units, generating oligonucleosomal DNA fragments of 180-200 bp. Agarose gel electrophoresis is a reliable method for identifying apoptotic cells in cell suspensions since DNA fragments create

a distinct ladder pattern upon electrophoresis. Necrotic cells are revealed in agarose gel electrophoresis as a smear. However, cell suspensions from oral epithelium takes more than two hours to prepare, which may be a time lapse, long enough to possibly skew the results. Therefore the terminal deoxynucleodidyl transferase (TdT)-mediated deoxyuridine triphophate (dUTP)-biotin nick end labeling (TUNEL) method (Gavrieli *et al.* 1992) on tissue sections was used. By this method DNA strand breaks are labeled.

Increasing numbers of keratinocytes with labeled DNA strand breaks were identified in the buccal epithelium of 5-FU treated animals, compared to controls. Hence, also these results were consistent with the theory of apoptosis. Yet, the results of end-labelling studies (TUNEL) are to be interpreted with caution, as some DNA strand breaks also in necrotic cells may be labeled, producing false-positive results (Grasl-Kraupp *et al.* 1995). Confirmation of the results has therefore been advocated.

(iv) Morphological assessment by ultrastructural analysis is an irrefutable means of identification of apoptosis (Cummings *et al.* 1997). By transmission electron microscopy (TEM) apoptotic cells appear with chromatin condensed as crescents around the nuclear envelope and apoptotic bodies can easily be identified. Necrotic cells are identified by cell membrane loss, and low cytocolic density. In the present ultrastructural analysis neither signs of necrosis, nor chromatin condensation around the nuclear envelope or apoptotic bodies were seen. Since chromatin condensation and apoptotic bodies are structural hallmarks of apoptosis, the assessment of the death process in the keratinocytes did not support the full range of features attributed to apoptosis.

The cells showed prominent cytoplasmic vacuoles. The morphological changes were seen in the cell layers closest to the basal lamina. The vacuoles were present in the perinuclear cytoplasm and progressed towards the perifery, with increasing exposure to 5-FU. When chromatin condensation was seen, it was confined to the central parts of the nuclei. The desmosomes could be seen, but the distances between them were larger in 5-FU treated animals than in untreated controls. The junctional complexes seemed to be lost to some extent. The keratinocytes in the basal cell layers were shrunk and the intercellular spaces widened. These findings, together with the other findings, are consistent with autophagic degeneration (Clarke 1990). Hence, it was concluded that the alterations in keratinocytes were compatible with autophagic degeneration.

Cell numbers by stereology

The cells of the basal cell layer of rat buccal epithelium have been estimated to have an average volume of 600 μm^3, which increases 30-fold by the time the cells reach the granular layer (Meyer *et al.* 1970). The results, when using the stereology method (I), were well in accordance with these estimates. In untreated control animals there were 1.25 cells per 1000 μm^3 of epithelium in the basal cell layer, in other words one cell per 800 μm^3.

Autophagic degeneration by 5-FU

The results of this study, featuring autophagic degeneration in the keratinocytes, were somewhat surprising since many studies on 5-FU report an apoptotic mode of cell death in different tissues (Inada *et al.* 1997; Lee 1993; Sakaguchi *et al.* 1994; Tong *et al.* Oncol 2000). In a recent *in vitro* study, however both apoptosis, necrosis and a third, mixed form was seen (Matsuo *et al.* 2001). A feature of cell death by apoptosis is the involvement of caspases. It has been proposed that cells, in which caspases are blocked, will die even though they do not acquire apoptotic morphology. These cells show features of autophagic degeneration. It is suggested that in these cells, lysosomal and other proteases are activated. In the study by Kitanaka it was proposed that autophagic degeneration does not involve caspase 3, the most central of the caspases (Kitanaka and Kuchino 1999). The level of active caspase 3 in our model system needs further investigation.

The p53 protein, involved in cell-cycle control and DNA repair, exerts its functions in the gap 1 (G_1) cell-cycle checkpoint, participating in preventing a cell with distorted DNA from progressing from G_1 to the synthesis (S) phase. P53 is expressed in very small amounts in cells under normal conditions. It is activated in response to DNA damage. The p53 protein contributes to the induction of apoptosis by 5-FU (Yukimoto *et al.* 2001), other cytotoxic drugs and irradiation (Patel *et al.* 2000). It has been reported that p53 may be crucial for 5-FU induced cell death (Benhattar *et al.* 1996). However, recently it was shown that other factors may act independently of p53 in inducing cell death by 5-FU (Mirjolet *et al.* 2000). This finding may imply another route for cell death by 5-FU, possibly in line with the present findings.

The results of autophagic degeneration in oral keratinocytes after 5-FU *in vivo* treatment were new. Further studies may clarify the fate of oral keratinocytes after 5-FU treatment and if p53 and caspase 3 are activated in the rat oral keratinocytes.

Inflammation

Autophagic degeneration is not a fully defined type of cell death. The studies in this thesis did not clarify if, at the end of the cell death process, the affected cells from 5-FU treated rats would be lysed, as in necrosis, or phagocytosed with intact cell membranes as in apoptosis. Even though the keratinocytes seemed to enter a programmed cell death pathway, sharing many features with apoptosis, it cannot be ruled out that the final phase may be necrotic. Autophagic degeneration has been suggested to be classified under necrosis, although the initial phases are considered as programmed cell death. In necrotic cells, the loss of plasma membrane integrity results in release of intracellular debris in the tissues, which in turn will elicit an inflammatory reaction.

When scattered cells die by apoptosis, the apoptotic bodies are phagocytosed by macrophages or neighbouring cells before plasma membrane integrity is lost (Savill *et al.* 1993), without eliciting an inflammatory reaction. However, this well-established theory has recently been challenged in a study in which macrophages produced pro-inflammatory cytokines after phagocytosis of apoptotic bodies (Kurosaka *et al.* 2001). If the final phase is apoptotic, the large quantity of contiguous cells that may be involved, may still preclude the possibility of phagocytosis, since there will not be enough neighbouring cells for phagocytosis. Then it is not unrealistic to propose that an inflammatory reaction may still occur and a mucositis may be elicited.

LOCAL IMMUNE DEFENCE

Dendritic cells (II, III)

A reduction of *the number of MHC class II molecule expressing cells* with dendritic appearance was seen in the epithelium and lamina propria of the buccal mucosa, as well as in the dental pulp. In the epithelium the decrease was seen only at high dose of 5-FU (II). The dendrites of the Langerhans cells of the buccal epithelium showed progressive degenerative features with increasing doses of 5-FU (II).

The *functional capacity of accessory cells* of both the buccal epithelium and the dental pulp to induce a ConA stimulated T cell proliferation was reduced after 5-FU treatment. The accessory capacity of the epithelial cells was reduced only after high dose of 5-FU treatment while in the dental pulp it was attenuated even after low dose treatment (III). The accessory function of epithelial or pulpal cell suspensions is carried out by MHC class II molecule expressing cells of these tissues. In the epithelium keratinocytes have been reported to be able to express MHC class II molecules under certain conditions. However, the cells in the epithelium, which were positive for MHC class II after immunohistochemical staining, showed a typical dendritic appearance in both untreated and 5-FU treated rats and were considered to be LC (II). Since macrophages are reported to outnumber DC in the dental pulp, it can not be ruled out that some of the accessory function performed by pulpal cells was carried out by macrophages. However, since the macrophages did not decrease in number in the pulp, these cells seemed to be less sensitive to 5-FU. Some of the accessory capacity could still have been affected.

The findings in these studies suggest that the MHC class II molecule expressing DC were affected by 5-FU, both in numbers and function. A local immunosuppression of the immune response of DC thus seemed to be in effect. It must be born in mind that the observed decrease may represent a decrease in actual number of cells, or a decrease in MHC class II molecule expression on the cells, or both.

Decrease in the number of MHC class II cells (II, III)

If a decrease in the actual number of cells occured in 5-FU treated animals, it is reasonable to assume that this mirrored a decreased supply of new DC from the bone marrow, since DC are bone marrow derived cells. The disappearance of cells from the tissues could theoretically be an effect of 5-FU on proliferating DC in the periphery. However, there is little evidence for DC peripheral division within the tissues. In a recent study, after an induced epidermal heat injury with total depletion of LC in mice, repopulation occurred from the circulation and increased dramatically after 5-7 days and reached normal density 11 days after injury (Ghaznawie *et al.* 1999a). No mitotic activity in the LC was registered in the peripheral tissue. Data thus suggested that the disappearance of DC from the tissues be due to senescence and apoptosis or evacuation of cells from the tissues to the lymph nodes. With an estimated turnover rate of 2-9 days it is reasonable to assume that the decrease in the number of DC 9

days after start of 5-FU treatment in the present study would, to some extent, be attributed to a decreased supply of these cells from the bone marrow.

Decrease in MHC class II molecule expression (II, III)

However, it can not be ruled out that a decrease of MHC class II molecule expression on remaining DC also occurred. This is supported by the findings that a suspension of lymph node cells from 5-FU treated rats, depleted of MHC class II expressing cells, after 3 days in culture contained some cells which displayed MHC class II molecules and a T cell proliferation was initiated (III). This would not be expected. In this experiment depletion of DC may not have been successful if there were DC in the initial cell suspension with a debilitated capacity to express MHC class II molecules, caused by 5-FU. 5-FU affects RNA and protein synthesis. It is tempting to speculate that MHC class II molecules, being glycoproteins, were affected by this 5-FU effect.

Decrease in function (III)

The impaired accessory capacity of the cells in epithelial (50 mg/kg 5-FU) or pulpal (30 mg/kg50; mg/kg 5-FU) cell suspensions to induce T cell proliferation (III) could reflect the combined effects of decrease in MHC class II molecule expressing cells, (whether by a decreased cell number or a decreased MHC class II expression) and an impaired capability of existing MHC class II molecules to function adequately. Increasing the number of epithelial cells from 5-FU treated rats in the cell suspensions did not restore the T cell proliferation (III). This may indicate that the function of MHC class II molecule expressing cells was debilitated after 50 mg/kg 5-FU treatment.

There was a difference in the accessory capacity between epithelial cells and pulpal cells, obtained from 30 mg /kg 5-FU treated rats (III). The number of LC also remained unchanged in the epithelium of 30 mg/kg 5-FU rats but not in the connective tissue and not in the dental pulp (II). These findings could possibly reflect an upgrading of LC capacity in the epithelium, related to an increased antigen exposure from the oral cavity in 5-FU treated rats. An elevated antigenic load of the oral epithelium compared to the dental pulp, with upregulated MHC class II molecule expression and cytokine expression, may explain the differences in capacity of accessory cells of the buccal epithelium and dental pulp to induce T cell proliferation in 30 mg/kg 5-FU rats.

At 50 mg/kg 5-FU, stimulation by an even higher bacterial load and further destruction of keratinocytes would most likely still not result in upregulated function of the DC, since the effect of the higher dose of 5-FU will have a detrimental effect on DC.

Degenerative features of LC (II)

The dendrites of the LC of the buccal epithelium showed progressive degenerative dendritic features after 8 days with increasing doses of 5-FU, adding to the picture of debilitated LC (II). These LC in the epithelium after 8 days of 5-FU high dose treatment could be remaining surviving cells. Despite short supply of bone marrow derived immunocompetent cells, they could also be some newly arrived LC, trying to develop to fully mature LC.

T lymphocytes (II)

CD2 positive cells (*T_H, CTL and NK cells*) decreased in number in the epithelium and in the lamina propria of the buccal mucosa and in the dental pulp, both at low and high dose (II).

The proportions of CD4/CD8 expressing cells, 75%/25%, in cell suspensions from the lymph nodes of control rats did not change after 5-FU treatment. From this observation it is concluded that there was no difference in sensitivity to 5-FU in these clones.

Macrophages (II)

ED2, a specific marker for macrophages, was used to identify these cells (Dijkstra *et al.* 1985). Macrophages of the dental pulp were not affected while the mucosal connective tissue macrophages showed a decrease in number. This may be attributed to differencies in the macrophages. During early phases of 5-FU treatment there may have been a recruitment to the oral mucosa of macrophages, due to increased antigenic load. These macrophages may react differently to 5-FU. Excudate macrophages are more sensitive than tissue macrophages, the latter being known to be fairly resistant. They do not proliferate for a long time, and may escape the effects of 5-FU. Studies on 5-FU effects on macrophages have shown that the drug appears to inhibit immature macrophages but not mature cells (Athlin and Domellof 1987; Connolly *et al.* 1983; Vetvicka *et al.* 1990).

Eosinophils and mast cells (IV)

Mast cells and occasional eosinophils were seen releasing their granules in the lamina propria of the buccal mucosa of 5-FU treated animals. It has been postulated that the mast cells do not release their contents or degranulate under normal physiological conditions. It can not be ruled out that these cells are involved in the inflammatory reactions of the oral mucosa after chemotherapy treatment. Because of the low mitotic activity of mast cells it is possible that mast cells are not destroyed by 5-FU treatment. This has to be further investigated.

MICROFLORA OF THE ORAL CAVITY AND INTESTINE (IV)

An increase in the number of facultative and strictly anaerobic bacteria in the oral cavity was found. There was also an increase in facultative anaerobic bacteria in the large intestine after 5-FU treatment. This is in line with other reports on an increased bacterial load in the intestine after 5-FU treatment. The number of facultative anaerobic gram-negative rods increased and gram-positive cocci decreased in numbers.

The increased numbers of obligate anaerobes and gram-negative rods in the oral cavity may be due to the decrease of gram-positive cocci, which were sensitive to 5-FU. The sensitivity by gram-positive cocci is in line with an early study which showed that a *Streptococcus* strain could be used for studies on sensitivity to 5-FU (Brandberg *et al.* 1977).

The tongue, which was used for anaerobic measurement, may harbour anaerobic bacteria in the papillary surface. It cannot be ruled out that an increase in the number of obligate anaerobes would have been found also in the intestine, if the samples of intestinal content had been taken from the intestinal mucosa instead of the lumenal content.

There are extensive studies showing the intense exchange of nutrients and waste products between intestinal lumen, the single layer columnar epithelium and underlying tissues. The exchange occurs through the epithelial cells, while permeation between the cells is limited, due to tight junctions. Also bacteria may enter endothelial cells or even disrupt tight junctions (Simonovic *et al.* 2001). In an injured intestinal epithelium the risk for bacterial invasion increases. Studies indicate, however, that also when the epithelium is injured the bacteria will enter through and not between the epithelial cells.

There are studies in recent years that have shown that bacteria may enter epithelial cells also in the oral epithelium. Hence *Porphyromonas gingivalis* can invade cells in an epithelial cell line via receptor-mediated endocytosis (Sandros *et al.* 1996). It has also been shown that such invasion will recruit immune defence cells. It cannot be ruled out that bacteria may invade keratinocytes also in other parts of the oral cavity.

BACTERIAL TRANSLOCATION TO CERVICAL AND MESENTERIC LYMPH NODES (IV)

The findings in the intestine, with an increased bacterial load (IV), injured epithelium (I, II) and impaired immune defence (II, III), may be prerequisites for bacterial translocation from the intestine to the mesenteric lymph nodes (Berg *et al.* 1988). It is not unreasonable to assume that translocation may also occur from the oral cavity to regional lymph nodes if corresponding alterations in barrier functions occur in the oral cavity. The finding that bacteria were identified in the cervical lymph nodes after 5-FU treatment was a new and interesting finding. In particular, facultative gram-negative rods increased in number. From the studies performed it can not be determined whether the increased number of bacteria in the lymph nodes reflects an increased translocation after 5-FU treatment or if bacteria, translocated already before treatment, proliferated during 5-FU treatment. Yet, it can be concluded, that the cervical lymph nodes are a route for bacterial spread from the oral cavity in range with bacterial spread from the intestine to the mesenteric lymph nodes. These findings reinforce the oral cavity, along with the gastrointestinal tract, as an important source for bacterial dissemination.

EFFECTS OF PROBIOTIC BACTERIA (IV)

Lactic acid bacteria are present in the intestine of most animals. The beneficial role played by these microorganisms in humans and animals, including the effect on the immune system, has been extensively reported.

In the intestine *L. plantarum* 299v to 5-FU treated animals lowered the numbers of facultative anaerobes compared to 5-FU treated rats, however the same was not seen in the oral cavity. The predominance of gram-negative rods that 5-FU had evoked both in the oral cavity and in the intestine was not normalised *by L. plantarum* 299v. In the cervical and mesenteric lymph nodes the total numbers of facultative anaerobes did not differ significantly between 5-FU treated animals and 5-FU treated animals receiving *L. plantarum* 299v.

5-FU treated rats supplemented with *L. plantarum* 299v lost less in weight, 12.1 % compared to 5-FU treated rats on tap water, $p<0.001$ and improved their food intake. The 5-FU induced diarrhoea, measured by liquid content of lumenal content, was not prevented by the administration of *L. plantarum* 299v. This points to the notion that it is not the total bacterial numbers or the numbers of facultative anaerobes that are crucial in 5-FU induced diarrhoea.

Taken together, *L. plantarum* 299v to some extent normalised 5-FU induced disturbances in the microbiota, but only in the intestine. It did not prevent bacterial dissemination to lymph nodes and did not improve diarrhoea. Yet, it improved the well being of the rats as measured by food intake and body weight. Further studies are needed to evaluate if *L. plantarum* 299v can have some positive outcomes in the efforts to hamper bacterial overgrowth in the oral cavity.

GENERAL DISCUSSION

Disrupted oral barrier functions by 5-FU

Cytotoxic drugs like 5-FU may cause oral mucositis. Mucositis is an inflammatory reaction in oral soft tissues, with the signs of inflammation, tumor (swelling) rubor (redness), dolor (pain) and calor (heat). Ulcerations, which sometimes also develop during treatment, are usually considered as a subsequent event to, or part of, a mucositis reaction. An inflammatory reaction can be induced by tissue injury or infection (Goldsby *et al.* 2000). In this thesis both tissue injuries and disturbed bacterial equilibrium were demonstrated in the oral cavity. Furthermore, the immune defence was debilitated, which may cause an invasion of bacteria with detrimental effects.

5-FU is rapidly transported into the cell by facilitated diffusion. Rapid metabolic elimination leads to a fall in 5-FU plasma levels with a primary half-life of 8 to 14 minutes (Grem 1990). The clinical signs of mucositis will however usually not occur until 7-10 days after treatment. An issue to be raised is, if clinical signs of cytotoxicity will take this long to develop. Another notion would be that the cell and tissue injuries develop very fast and the signs of mucositis are due to a secondary infection, which will take 7-10 days to develop. In other words, is it the primary tissue injury by cytotoxicity, or a secondary infection, which elicits the clinical signs of mucositis.

Tissue injury by cytotoxicity

A comparison of oral epithelium to other tissues may give some indication on how fast the clinical signs of cell injury by 5-FU may arise. WBC have a nadir count 6-8 days after a single dose of 5-FU (Friberg *et al.* 2000; Simonsen *et al.* 2000). Since bone marrow cells have a turnover rate that is higher than epithelial cells it seems reasonable to assume that effects of cytotoxicity on keratinocytes will not display until several days after treatment. The results from study I showed that the keratinocytes degenerate by 5-FU and that more cells were affected after 3 days than after 1 day. After 3 days, the cell layers closest to the basement membrane were affected while cells higher up in the epithelium still seemed rather unaffected (I). These findings support the theory that it will take several days for keratinocytes affected by cytotoxicity to reach the epithelial surface.

The changes in the oral keratinocytes were consistent with autophagic degeneration. If the cell remnants in autophagic degeneration are phagocytosed like apoptotic cells, inflammation is not expected. If there is a lack of adjacent viable cells capable of phagocytosing the apoptotic bodies an inflammatory reaction might still develop. If the end product of autophagic degeneration is necrosis, an inflammatory reaction will certainly be elicited, if there are enough inflammatory cells. A tissue injury may induce a complex cascade of events in the inflammatory reaction, including vasodilation (Goldsby 2000) and leaking of fluid. This leads to accumulation of fluid in the tissues, oedema, with tumor, rubor and calor as part of the inflammatory reaction.

Infection

If the main cause of mucositis is infection the scenario may be the following. The epithelial cells die rather quickly by autophagic degeneration. With a high turnover rate in epithelium, the oral epithelial barrier function may be deteriorating by cytotoxicity within days, enough for the increased amount of bacteria in the oral cavity, shown in study IV, to gain access to the tissues, and infection may be the result. Also in this case, a cascade of events in the inflammatory reaction, including vasodilation and leaking of fluid, will occur, causing mucositis symptoms.

Both deteriorating keratinocytes, and invading bacteria or toxins, will elicit cytokine expression, which will in turn contribute to an inflammatory reaction.

Other factors

When epithelial cells degenerate and die following anticancer treatment the epithelium becomes thinner and detaches (II). The oral mucosa is constantly exposed to mechanical forces by mastication, which increases the risk of ulcerations in the attenuated epithelium. Based on clinical experience, not yet scientifically studied, it is evident that ulcerations are often found in the lining buccal mucosa (Squier 1990) at the level of the upper molars, inside the corner of the mouth, inside the lower lip or in the floor of the mouth. The ulcers may develop as a consequence of a thinner epithelium and mechanical trauma on surfaces especially exposed.

The normal epithelial thickness may also be of importance. The floor of the mouth and the ventral tongue have a thinner epithelial layer (170 μm) than the buccal mucosa (767 μm) (Chen and Squier 1984). The buccal mucosa in turn is nonkeratinised in humans while the epithelium on the dorsum of the tongue and palate have a keratinised layer. The buccal mucosa of rats is orthokeratinised. Interestingly, the rats did not show clinical signs of mucositis (II) although both the keratinocytes, the bacteria and the immunocompetent cells were disturbed in the current rat model. It cannot be ruled out that the keratinised layer served some protective function.

The differences in epithelial cell proliferation activity at different locations in the oral cavity may be another factor. Cells with a high turnover rate are more likely to be affected by 5-FU. Thomson et al have shown that cells undergoing DNA synthesis in human oral tissues, measured by percentage of cells within the S phase of the cell cycle, were highest in the floor of the mouth and ventral tongue while activity was lowest in the dorsum of tongue and the palate (Thomson *et al.* 1999). The buccal mucosa had a higher number of cells in S phase than mandibular gingiva (Thomson *et al.* 2001).

An additional load of cytotoxicity may also be anticipated, from cytotoxic drug supplied from the major salivary glands, since it has been shown that 5-FU is secreted in the saliva (Oliff *et al.* 1979). This will particullary expose the lining mucosa around the orifice of the salivary gland ducts, i.e. the buccal mucosa and the floor of the mouth.

Saliva composition and quantity were not investigated in this thesis. However, it deserves to be mentioned. Many patients experience a feeling of roughness on oral mucosal surfaces during 5-FU treatment. Changes in salivary quantity and quality may cause secondary effects since the saliva has both a protective and lubricating function on oral mocosa, as well as taking part in food processing. Reduced salivary flow, which has been recorded as a risk factor for developing mucositis during 5-FU treatment (McCarthy *et al.* 1998) or impaired quality, make mucosal surfaces more vulnerable to mechanical forces and may facilitate bacterial colonisation.

The capacity of individuals to enzymatically degrade 5-FU by DPD (the rate limiting enzyme dihydropyrimidine dehydrogenase) may vary. It is possible that the increased tendency in some patients to develop oral mucositis may be due to a deficiency in DPD in epithelial cells.

Multifactoral aetiology

Mucositis is a clinical diagnosis based on symptoms. As in many biological events there may be a series of competing factors leading to the clinical symptoms. In fact, all these factors may work in concert, the vascular changes, degenerating keratinocytes and attenuated epithelium, bacterial imbalance and affected immune response, salivary changes as well as individual factors in the development of mucositis. It has been suggested that the condition should be better referred to as mucosal barrier injury (MBI), with the notion that it is a complex and dynamic pathobiological process manifested not only in the mouth but also throughout the entire digestive tract (Blijlevens 2000).

Different cytotoxic drugs may affect the various factors described above differently, depending on the type of effect on a cellular level. It may be speculated that only one, or a few of the phenomena, may arise in some cases or that all are in action but only one or a few are dominating. They may run in parallel or in a specific sequence that finally will be clinically revealed as mucositis. Indeed, it is likely that they interact.

Four phases have been proposed regarding the mechanisms by which mucositis, induced by cytotoxic drugs, develops and heals: inflammatory/vascular phase, epithelial phase, ulcerative/bacteriological phase and healing phase (Sonis 1998). To take this classification further, the concept of *compartments* is hereby proposed in the context of oral mucositis. Cancer chemotherapy is the cause but involves individual compartments differently in different patients. This model opens the possibility that one compartment is involved at several steps during the development of mucositis. A salivary compartment has been included in the model and the inflammatory/vascular phase has been separated to an immunological and a vascular compartment. The following classification is proposed, based on current knowledge on oral mucositis by cancer chemotherapy (Table 2). Note that the order in Table 2 does not imply a fixed order of appearance. Of the compartments, this thesis deals with (c) the keratinocyte/epithelial compartment (I), (d) the immunological compartment (II, III) and (f) the bacterial compartment (IV).

The compartments represent different barrier functions. Every compartment will include many *components* of cellular and biochemical events. In the course of mucositis development, a compartment will be involved at different levels with different components. The different components may be outlined according to Tabel 3 (compartment in brackets).

In the rat model, it was also shown that bacteria may be found passed the major barrier functions after 5-FU treatment, in the cervical as well as the mesenteric lymph nodes. This was an important finding, in the ongoing discussion on the oral cavity as a source for bacterial spread and dissemination of infections.

Table 2. *Compartments* of events during the course of mucositis, caused by cancer chemotherapy

(a) Drug distribution compartment
(b) Vascular compartment
(c) Keratinocyte/epithelial compartment
(d) Immunological compartment
(e) Salivary compartment
(f) Bacterial compartment
(g) Healing compartment

Table 3. An outline of the different *components* during the course of mucositis, caused by cancer chemotherapy. Compartments are indicated in brackets.

↓ Treatment and drug distribution (a)
↓ Interaction with hematopoetic cells (d)
↓ Interaction with keratinocyte cell cycle/autophagic degeneration (c)
↓ Cytokine release from keratinocytes (c)
↓ Vascular endothelial permeability increase/migration of immunocompetent cells/oedema (a, b, d)
↓ Recruitment of innate immunocompetent cells (d)
↓ Cytokine release from immunocompetent cells (d)
↓ Activation of specific immune response (d)
↓ Saliva affected (flow, composition) (e)
↓ Drug interaction with bacteria/distortions in bacterial balance (f)
↓ Bacterial interaction with epithelium/colonisation (f)
↓ Decreased number/downregulation of immunocompetent cells (d)
↓ Cytokine release from keratinocytes and bacteria (c, f)
↓ Morphological changes in the whole epithelium, increased permeability (c)
↓ Bacterial invasion (f)
↓ Bacterial translocation (f)
↓ Drug elimination and healing (g)

CONCLUSIONS

- 5-FU *in vivo* treatment did not cause a typical apoptotic mode of cell death in the keratinocytes of the epithelium of the buccal oral mucosa. 5-FU treatment induced alterations that are consistent with autophagic degeneration. The changes were confined to the cell layers closest to the basement membrane after a 3-day treatment (I).

- A reduction in the number of MHC class II molecule expressing cells with dendritic appearance (DC) was seen in the epithelium and lamina propria of the buccal oral mucosa and in the dental pulp. In the epithelium, the decrease was seen only after 5-FU high dose treatment. The dendrites of the Langerhans cells of the buccal epithelium showed degenerative features. T cells decreased in numbers both in the oral mucosa and dental pulp. Macrophages decreased in number in the lamina propria of the oral mucosa (II).

- The capacity of accessory cells of the buccal oral epithelium to induce a conA stimulated T cell proliferation was decreased after 5-FU high dose treatment. The capacity of accessory pulpal cells to induce a conA stimulated T cell proliferation was decreased at both low and high dose treatments. An elevated antigenic load of the oral epithelium, compared to the dental pulp, with upregulated MHC class II molecule expression and cytokine expression, may explain the differences in capacity of accessory cells of the buccal epithelium and dental pulp to induce T cell proliferation (III).

- 5-FU caused an increase in the numbers of facultative and strictly anaerobic bacteria in the oral cavity and in the number of facultative anaerobes in the intestine. The proportions of facultative gram-negative rods increased (IV).

- The cervical lymph nodes seemed to be an important route for spread of bacteria from the oral cavity during 5-FU treatment, on a level with the mesenteric lymph nodes. This finding reinforces the oral cavity, along with the gastrointestinal tract, as a source for bacterial dissemination (IV).

- The use of a probiotic bacterium, *L. plantarum* 299v, may to some extent normalise 5-FU induced bacterial disturbances in the oral cavity and intestine. However, *L. plantarum* 299v did not decrease the total bacterial numbers in the lymph nodes of 5-FU treated animals (IV).

- The use of *L. plantarum* 299v improved the well being of 5-FU treated animals by improved food intake and increased body weight compared to rats on 5-FU alone. *L. plantarum* 299v did not improve 5-FU induced diarrhoea (IV).

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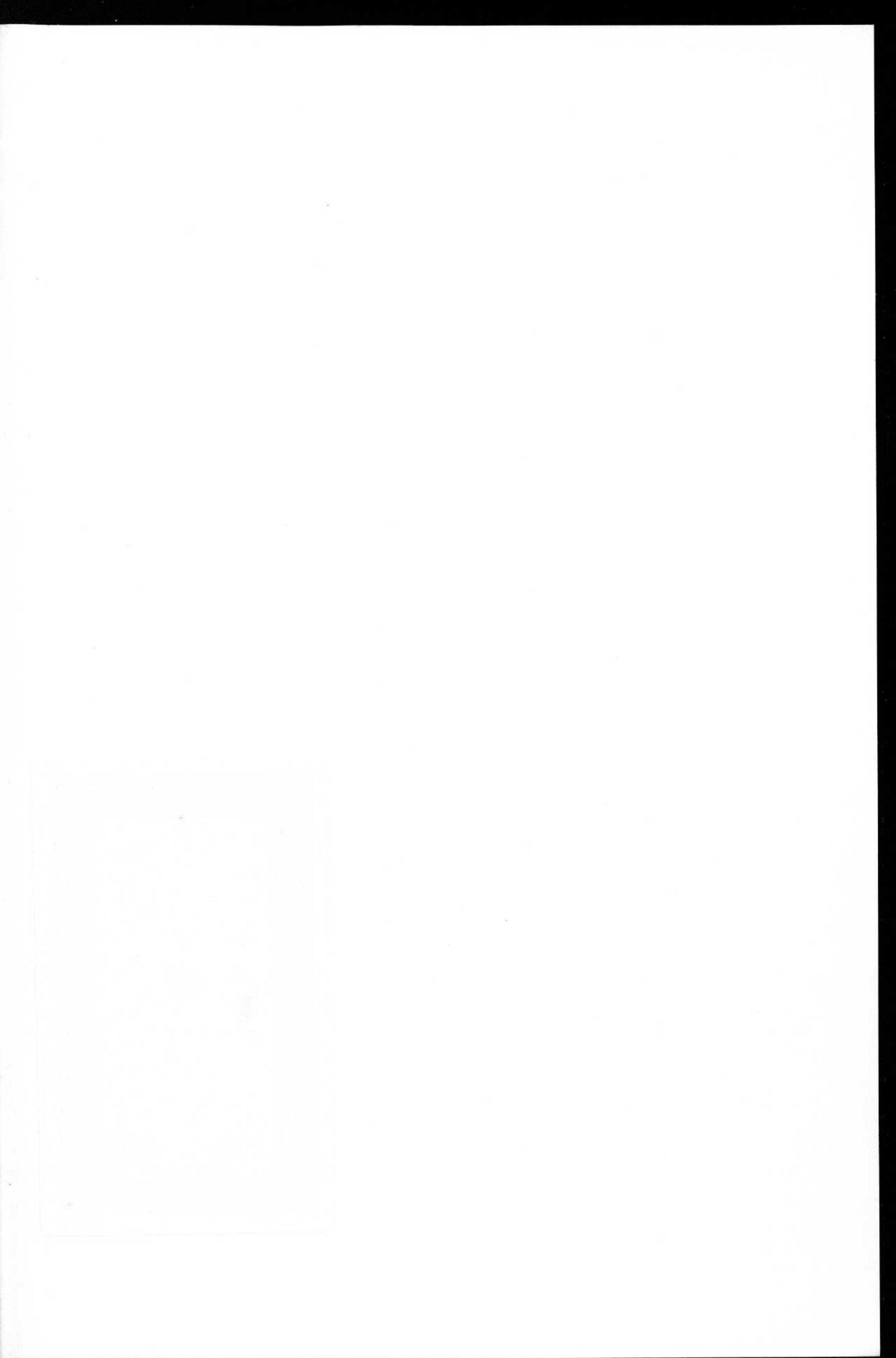

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