



Université Catholique de Louvain
Faculté de Médecine
Service de Gastro-entérologie

**CELIAC DISEASE AND
CHOLECYSTOKININ CELL DYSFUNCTION:
A MODEL OF INTERACTION BETWEEN THE DIGESTIVE,
ENDOCRINE AND IMMUNE SYSTEMS IN THE GUT.**

Pierre H. Deprez

Thèse présentée en vue de l'obtention du Grade de Docteur en Sciences biomédicales

Orientation : Gastro-Entérologie

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Summary	1
Résumé	3
Abbreviations	5
Chapter I Introduction	7
Chapter II Cholecystokinin	9
1-General overview	9
2-Peptide structure	10
3-Biosynthesis and processing	11
4-Cellular/molecular mechanisms regulating CCK cell differentiation	12
5-Cellular/molecular mechanisms regulating CCK gene expression	13
5.1 CCK gene and promoter	13
5.2 CCK gene expression and nutrients	13
5.3 CCK gene expression in STC-1 cell line	14
5.4 Neuropeptides and PACAP	14
6-CCK-1 and CCK-2 receptors	14
7-CCK receptor antagonists	15
8-Regulation of secretion	18
8.1 Effects of nutrients on CCK secretion	19
8.2 Feedback regulation of CCK secretion	19
8.2.a Feedback regulation in rats	19
8.2.b CCK releasing peptides	19
8.2.c Feedback regulation in men	20
8.2.d Feedback regulation and surgery	20
8.3 Neural regulation of CCK secretion	20
8.4 CCK release in the brain	20
9-Biological functions of CCK in the alimentary tract	21
9.1 Pancreas	21
9.1.a Pancreatic secretion	21
9.1.b Pancreatic growth	22
9.1.c Regulation of carbohydrate metabolism	22
9.1.d Clinical impact in pancreatic diseases	22
9.2 Gallbladder and bile ducts	23
9.2.a Gallbladder	23
9.2.b Sphincter of Oddi	23
9.2.c Clinical impact in biliary diseases	23
9.3 Stomach	24
9.3.a Gastric emptying	24
9.3.b Regulation of acid secretion	24
9.3.c Functional dyspepsia	25
9.4 Motility effects on the oesophagus	25
9.5 Motility effects on the small intestine	25
9.6 Motility effects on the colon	26
9.7 CCK as a growth factor	26
9.7.a Pancreas	26
9.7.b Pancreatic cancer	26
9.7.c Other cancers	27
9.7.d CCK-2 receptors and gastrin	27
9.8 Various other effects	27
9.8.a Pepsinogen secretion	27
9.8.b Somatostatin release	27
9.8.c Pancreatic polypeptide release	27

10-Biological functions of CCK in the brain	28
10.1 General overview	28
10.2 CCK Receptor Subtypes, agonists and antagonists	28
10.3 Role of CCK in analgesia	29
10.4 Role of CCK in memory	29
10.5 Role of CCK in satiety	29
10.6 Role of CCK in anxiety	31
10.7 Summary	32
11-Causes of deficiency	32
11.1 Celiac disease	32
11.2 Bulimia, and various diseases associated with reduced CCK	32
11.3 Autoimmune polyglandular syndrome type I	32
11.4 CCK and CCK-receptors knockout mice	33
Chapter III Celiac disease	35
1-Epidemiology	35
2-Pathophysiology	35
2.1 Abnormal permeability	35
2.2 Genetic predisposition	36
2.3 Interaction between the tTG modified gliadin peptide, HLA-DQ2 and T cells	36
2.4 Events leading to mucosal lesions	36
2.4.a Morphological types of mucosal lesions	36
2.4.b Involvement of T cells	37
2.4.c Role of intraepithelial lymphocytes (IELs)	38
-General description and function	38
-“Type a” IELs	38
-”Type b” IELs	39
-IELs and celiac disease	39
-Functions of IELs	39
-Gene-expression profile of IELs	39
2.4.d Epithelial cell apoptosis	40
3-Clinical Manifestations	40
3.1 Celiac Sprue in Children	40
3.2 Celiac Sprue in Adults	40
3.3 Associated Conditions	41
4-Diagnosis	41
4.1 Serologic Tests	41
4.2 Haematological and Biochemical Tests	41
4.3 Biopsy of the Small Intestine	41
4.4 Repeated Biopsy and Gluten Challenge	42
4.5 Genetic testing	42
5-Treatment	42
6-Complications: refractory sprue and lymphoma	43
7-CCK deficiency and pancreatico-biliary dysfunction	43
Chapter IV Persistent decreased plasma cholecystokinin levels in celiac patients under gluten-free diet: respective roles of histological changes and nutrient hydrolysis	45
1-Introduction	45
2-Methods	45
2.1 Subjects	45
2.2 Test meal	46
2.3 Sampling and extraction of cholecystokinin	47
2.4 Cholecystokinin radioimmunoassay	47
2.5 Gallbladder volume measurement	47
2.6 Statistical analysis	47

3-Results	48
3.1 Patients data	48
3.2 Basal CCK immunoreactivity	48
3.3 Postprandial plasma CCK levels	49
3.4 Gallbladder volumes	49
4-Discussion	51
Chapter V Expression of cholecystokinin in the duodenum of patients with celiac disease: respective role of atrophy and lymphocytic infiltration.	55
1-Introduction	55
2-Methods	55
2.1 Patients	55
2.2 Immunocytochemistry	56
2.3 Tissue extraction and radioimmunoassay	56
2.4 Semiquantitative RT-PCR	56
2.5 Statistical analysis	57
3-Results	57
3.1 Patients	57
3.2 Immunocytochemistry	58
3.3 Tissue extraction and radioimmunoassay	58
3.4 Semiquantitative reverse RT-PCR	59
4-Discussion	59
Chapter VI Modulation of cholecystokinin release by cytokines on short-term cultured duodenal biopsy samples and on STC1 cells.	63
1-Introduction	63
2-Methods	63
2.1 Patients	63
2.2 Chemicals	64
2.3 Cell culture	64
2.4 Organ culture	64
2.5 IEL and LPL supernatants	64
2.6 Release studies	64
2.7 CCK extraction and RIA	65
2.8 Statistics	65
3-Results	65
3.1 STC-1 cells	65
3.1.a Basal conditions	65
3.1.b Releasing studies	65
3.2 Organ culture	66
3.2.a Viability of organ culture	66
3.2.b Basal conditions	66
3.2.c Releasing studies	67
4-Discussion	67
Chapter VII Synthesis and conclusions	69
Chapter VIII Perspectives	71
References	72
Author contribution relevant to the redaction of the thesis	94



Celiac sprue is the principal disease associated with impaired cholecystokinin (CCK) release that accounts for the diminished gallbladder contraction and pancreatic function. Celiac sprue, also known as celiac disease or gluten-sensitive enteropathy, is an autoimmune disorder characterized by malabsorption resulting from inflammatory injury to the small intestine mucosa after the ingestion of wheat gluten or related rye and barley proteins.

The aim of our work was to study the defective CCK release that is found in adult patients with celiac disease.

The CCK decreased secretion was first thought to be caused by a reduction in the number of intestinal CCK cells in the presence of mucosal atrophy, but could also be related to other mechanisms: a lack of stimulation of the mucosal CCK cells due to impaired intra-duodenal hydrolysis of nutrients, a functional abnormality of the CCK-cell, an impaired interaction of the CCK-cell with other intestinal cells (endocrine, epithelial or nervous cells), the absence of a stimulating factor (luminal cholecystokinin releasing factor) or the presence of inhibitory factors associated with the inflammatory process taking place in the mucosa.

We have shown that CCK release is not only impaired in untreated celiac patients with a destructive/hypoplastic type duodenal mucosa but also in patients whose mucosa solely displayed a significant intraepithelial lymphocytic (IEL) infiltrate. Only treated celiac patients without hypoplastic or destructive mucosa and without any increase in IEL infiltrate have a basal and postprandial CCK release similar to the control group. When using immunocytochemistry and semi-quantitative PCR we have demonstrated that the defective release of CCK observed in patients with coeliac disease is not related to a decrease in the number of CCK cells present in the proximal part of the small intestine but rather to a decrease in CCK synthesis related to a decrease in mRNA content, even in patients with an infiltrative type of celiac disease. These data suggest that the inflammatory infiltrate in the mucosa of celiac patients affects expression of the CCK gene.

The next part of our work was to investigate the role of deficient intraluminal nutrient hydrolysis in adult celiac patients. Our data indicates that ingestion of a pre-digested meal does not correct the defective CCK release in patients with a destructive or an infil-

trative type of celiac mucosal lesion. Moreover intraduodenal food digestion should be considered normal in celiac patients with mucosal lesions limited to an intraepithelial lymphocytic infiltrate. The decreased levels of plasma CCK in this group of patients, given a pre-digested meal, strongly support that the defective CCK release could be related to suppressive factors released by the inflammatory infiltrate.

We therefore studied the effects of supernatants obtained from IEL and lamina propria lymphocyte cultures and various cytokines produced by mucosal lymphocytes on short-term organ cultures of duodenal biopsy specimens obtained from normal controls and on the CCK secreting murine neuroendocrine tumour cell line STC-1. We demonstrated a significant inhibitory effect of supernatants obtained from phytohaemagglutinin A-stimulated cultured lamina propria lymphocytes and from phytohaemagglutinin A-stimulated co-cultured lamina propria and intraepithelial lymphocytes, and of interleukin-1 and tumour necrosis factor- α on CCK release.

All together these data show that the defective CCK release is present at the initial infiltrative type of mucosal lesion seen in celiac disease and that this defective release may be related to inflammation and suppressive factors associated with the inflammatory cells. Modulation of CCK release in the gut should be considered as a complex process implying co-regulation of nutrients, releasing peptides, neurotransmitters and soluble factors including cytokines. These findings may help to find new directions for improving treatment of patients with celiac disease who continue to present with abdominal complaints under a strict gluten-free diet.



La maladie cœliaque est la principale pathologie liée à un déficit en cholécystokinine (CCK) occasionnant une diminution de la fonction pancréatique et une réduction de la contraction vésiculaire. La maladie cœliaque ou intolérance au gluten est une affection auto-immunitaire qui se caractérise par une malabsorption intestinale causée par une atteinte inflammatoire de l'intestin grêle, suite à l'ingestion de gluten présent dans la farine de blé, d'orge ou de seigle.

Le but de ce travail est d'étudier la raison de ce déficit en CCK chez des patients adultes souffrant de maladie cœliaque.

La diminution de sécrétion de CCK a d'abord été attribuée à une réduction du nombre de cellules à CCK intestinales et de leur contenu hormonal dans le cadre d'une muqueuse intestinale atrophique, de type destructif ou hypoplasique. Elle pourrait cependant être liée à d'autres mécanismes: une diminution de la stimulation des cellules endocrines suite à une moindre hydrolyse des nutriments dans la lumière intestinale, un trouble fonctionnel des cellules, une altération de l'interaction avec d'autres cellules intestinales (endocrines, épithéliales ou nerveuses), l'absence d'un facteur stimulant la sécrétion de CCK ou la présence de facteurs inhibiteurs associés au processus inflammatoire dans la muqueuse intestinale.

Nous avons montré que la sécrétion de CCK n'est pas seulement diminuée chez les patients cœliaques présentant une muqueuse duodénale de type hypoplasique ou destructif mais également chez les patients dont la muqueuse ne présente qu'un infiltrat excessif de lymphocytes intraépithéliaux (IEL). Seuls les patients cœliaques traités, sans caractère destructif ou hypoplasique de leur muqueuse, et sans infiltrat inflammatoire, présentent une sécrétion de CCK normale et semblable aux volontaires sains. En utilisant l'immunocytochimie et la PCR semi-quantitative, nous avons démontré que le déficit en sécrétion de CCK n'est pas lié à une diminution du nombre de cellules à CCK dans la muqueuse duodénale mais plutôt à une diminution du taux d'ARN messager, et de la synthèse de CCK, ceci tant chez les patients avec des lésions muqueuses de type destructif ou hypoplasique que chez les patients ne présentant qu'un infiltrat de lymphocytes intraépithéliaux. Ces données suggèrent que l'infiltrat inflammatoire affecte l'expression du gène de la CCK dans les cellules entéroendocrines intestinales.

La partie suivante de notre travail a consisté à investiguer le rôle d'un déficit d'hydrolyse intraduodénale des nutriments sur la sécrétion de CCK chez des patients cœliaques adultes. Nous avons montré que la

sécrétion postprandiale de CCK n'est pas améliorée par l'ingestion d'un repas prédigéré, chez des patients présentant des lésions destructives ou hypoplasiques comme ceux présentant des lésions muqueuses infiltratives. De plus l'hydrolyse alimentaire intraduodénale devrait être normale en cas de lésion muqueuse limitée à un infiltrat de IEL. Les taux plasmatiques réduits de CCK dans ce groupe de patients suggèrent clairement que le déficit de sécrétion de CCK pourrait être lié au processus inflammatoire et à des facteurs provenant de l'infiltrat de lymphocytes intraépithéliaux.

Pour cette raison, nous avons étudié les effets de surnageants de cultures lymphocytaires et de cytokines produites par les lymphocytes de la muqueuse intestinale sur des biopsies duodénales maintenues en culture de courte durée et sur des cellules sécrétant de la CCK et provenant d'une lignée cellulaire murine neuroendocrine STC-1. Nous avons montré un effet inhibiteur significatif sur la sécrétion de CCK du surnageant de cultures de lymphocytes de la lamina propria stimulés par de la phytohémagglutinine A, ainsi que de l'interleukine-1 et du TNF- α .

En conclusion, nous avons montré que le déficit de sécrétion de CCK est présent dès le stade initial infiltratif de la lésion muqueuse cœliaque et que ce déficit pourrait être lié au processus inflammatoire présent dans la muqueuse intestinale et à certains facteurs suppresseurs associés à ces cellules inflammatoires. La modulation de la sécrétion de CCK doit être considérée comme un processus complexe impliquant une co-régulation par les nutriments, des peptides stimulants, des neurotransmetteurs et des facteurs solubles comme les cytokines. Les données décrites dans ce travail nous font entrevoir de nouveaux mécanismes de régulation et de nouvelles pistes pour améliorer la prise en charge de patients cœliaques continuant à présenter des troubles digestifs malgré un régime sans gluten bien suivi



AP₁	Activator protein 1
bHLH	Basic helix-loop-helix protein
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
CRE	Calcium response element
CREB	Calcium/3,5'-monophosphate response element binding protein
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
FCS	Fetal calf serum
GABA	Gamma amino butyric acid
GORD	Gastro-oesophageal reflux disease
HLA	Human leukocyte antigen
5HT	5 hydroxytryptamin
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IEL(s)	Intra-epithelial lymphocytes
IFN	Interferon
IgA	Immunoglobulin A
IL	Interleukin
IP₃	Inositol 1,4,5-triphosphate
LCRF	Luminal CCK-releasing factor
LOS	Lower oesophageal sphincter
LPL	Lamina propria lymphocytes
mRNA	Messenger ribonucleic acid
MHC	Major histocompatibility complex
OLETF	Otsuka Long Evans Tokushima Fatty
PACAP	Pituitary adenylate cyclase-activating polypeptide
PHA	Phytohaemagglutinin A
PKC	Protein kinase C
RIA	Radioimmunoassay
RT-PCR	Reverse transcription-polymerase chain reaction
TCR	T cell receptor
tTG	Tissue transglutaminase
TLOS_R	Transient lower oesophageal sphincter relaxation
VIP	Vasoactive intestinal polypeptide



INTRODUCTION

Cholecystokinin has been described as an important digestive hormone in the 1920s and is now associated with more than 13900 medical and scientific publications. Although its biological functions are well described and accepted in the alimentary tract and more recently in the brain, the actual mechanisms that lead to hormonal and neural regulation of these functions are not fully understood. Moreover, a variety of assumptions have been made concerning the sensory ability of gastroenteric cells on the basis of hormone release in response to various nutrients. There is no unequivocal evidence for direct stimulation of intestinal endocrine cells by nutrients. It is still unknown whether stimulation occurs through the apical or basolateral surface of the endocrine cells, and whether other epithelial cells, such as brush cells play a critical role in nutrient sensing.

The cellular and molecular mechanisms regulating CCK cell differentiation and gene expression are now better understood with the characterization of transcription factors associated with enhancers in the CCK gene. Recent knowledge about CCK both in the gut and in the brain will be discussed in chapter II.

Celiac disease is the main disease associated with impaired cholecystokinin release that accounts for the diminished gallbladder contraction and pancreatic function. Celiac disease is characterized by malabsorption resulting from inflammatory injury to the mucosa of the small intestine after the ingestion of wheat gluten or related rye and barley proteins. Celiac sprue has been defined more than 30 years ago in terms of the flat lesion. Recently, more refined descriptions of gluten-induced mucosal changes have been given that might be termed the spectrum of gluten sensitivity. Even in histological changes of lesser degrees, some functional abnormalities have been reported. Chapter III summarizes the epidemiology, pathophysiology, clinical diagnosis and treatment of celiac disease with special emphasis on digestive complications.

Celiac disease accounts for more than 14960 reports and of these, 71 publications refer both to celiac disease and cholecystokinin. Cholecystokinin deficiency has mainly been described in patients with a flat mucosa. The mechanism by which CCK release is impaired in celiac disease is still a matter of discussion. The number of CCK cells and their hormonal content were shown to be decreased in the presence of mucosal atrophy. Some immunocytochemistry reports however reported increased number of CCK cells and increased CCK immunoreactive content in

the celiac mucosa. Defective release or synthesis may therefore be related to other mechanisms: a lack of stimulation of the mucosal CCK cells due to impaired intra-duodenal hydrolysis of nutrients, a functional abnormality of the cell, an impaired relation with other intestinal cells, absence of a stimulating factor (e.g. luminal cholecystokinin releasing factor) or the presence of inhibitory factors associated with the inflammatory process taking place in the epithelium and the mucosa.

The aim of this thesis was to study CCK release in celiac patients with a large spectrum of lesions, i.e. celiac patients presenting with a destructive or infiltrative type mucosa or with a completely restored mucosa under gluten-free diet. We were able to show decreased plasma CCK levels in patients with a destructive or hypoplastic type of mucosal lesion but also in patients with infiltrative type (chapter IV). We therefore studied the relationship between the distinctive mucosal patterns observed in celiac disease and the mucosal CCK and CCK mRNA content. We hypothesized that if impaired nutrient hydrolysis was the limiting factor for CCK release, it would only be present in patients with an atrophic mucosa, i.e. destructive and/or hypoplastic. We therefore compared the effects of a polymeric or a semi-elemental liquid test meal on CCK release in controls and in the different groups of celiac patients (chapter V). Finally, to evaluate the immuno-endocrine interactions, we examined the role of the mucosal inflammatory infiltrate on CCK release from a murine neuroendocrine tumour cell line STC-1 and from short-term organ cultures of duodenal biopsy specimens (chapter VI).



CHOLECYSTOKININ

Cholecystokinin was described in 1928 by Ivy & Oldberg as a contaminant of impure secretin preparation that caused contraction of the dog and cat gallbladder (Ivy 1928). A pancreatic enzyme-stimulating activity found in 1943 by Harper and Raper suggested that cholecystokinin stimulates the secretion of pancreatic enzymes (Harper 1943). In 1971, Mutt and Jorpes purified and sequenced a hormone, able to stimulate both pancreatic enzyme secretion and gallbladder contraction (Mutt 1968). The first observation that CCK was present in the brain was not until 1975, followed a few years later by the isolation and sequencing of CCK 8 from sheep brain (Dockray 1978). This hormone was first called cholecystokinin-pancreozymin and finally just cholecystokinin (CCK), by historical precedent in its description (Grossman 1970).

1-General overview

CCK has proved to have several activities in addition to the two that led to its discovery. The hormonal effects are due to CCK in plasma that originates almost entirely from I cells in the small intestinal mucosa. CCK peptides are also expressed in large quantities in neurons, but neuronal CCK contributes negligibly to CCK in plasma. The chemical

structure of CCK is complicated by the natural occurrence of multiple molecular forms, which all play important roles in nutrition, food regulation and satiety signalling. We will review the biosynthesis and structure of this family of peptides, the CCK receptors and antagonists, the digestive and nutritional functions with their respective clinical impact, the satiety control and some psychobehavioural aspects exerted by the CCK “family” of peptides. The various clinical disorders possibly involving CCK are summarised in **Table 1** (Jensen 2002).

Table 1

CLINICAL DISORDERS LIKELY INVOLVING CCK

CCK deficient states	Celiac disease Bulimia Autoimmune polyglandular syndrome type 1 Diabetes mellitus Familial amyloid associated polyneuropathy Short bowel syndrome
Pancreatic disorders	Acute and chronic pancreatitis
Gastrointestinal motility disorders	Gallbladder disease Irritable bowel syndrome Functional dyspepsia Chronic constipation
Tumour growth Satiety, anxiety, memory disorders	Colon, gastric, pancreatic, liver
Decreased brain concentrations Huntington's disease	Schizophrenia

Adapted from Jensen 2002.

2-Peptide structure

Multiple molecular variants have been described since the original purification of the 33-amino-acid peptide in porcine small intestine by Victor Mutt. The carboxyl-terminal pentapeptide amide sequence of CCK was found to be identical to that of gastrin. This similarity in structure accounts for biologically overlapping activities on some receptors and for cross-reactivity between antibodies raised against either gastrin or CCK (Rehfeld 1978).

Intestinal extracts contain a 8-amino-acid form (CCK-8), a 33-amino-acid form (CCK-33), a 39-aminoacid peptide (CCK-39) and mostly a larger form containing 58 amino acids (CCK-58) (Dockray 1977, Eysselein 1982, Reeve 1991). The largest bioactive form is CCK-83, which can be extracted from very fresh intestine and corresponds to the amidated sequence 1–83 of pro-CCK (Eberlein, G.A. 1992). Its presence indicates the site of signal peptidase cleavage of preprocholecystokinin (Reeve 1986). This sequence is cleaved at five or more monobasic sites to release CCK-58, -39, -33, -22, and -8, all of which have

the same C-terminal heptapeptide amide sequence (-Tyr(SO₃⁻)-Met-Gly-Trp-Met-Asp-PheNH₂), that also is the minimal epitope necessary for receptor binding. Binding to CCK-1 receptors requires that the tyrosyl residue of the heptapeptide amide is O-sulphated, whereas CCK-2 receptors do not discriminate sulphated from non-sulphated CCK peptides or CCK from gastrin peptides. Other forms have also been isolated from rat intestine and brain (CCK-5, CCK-22) (Eng 1984). The peripheral and central neural forms consist mainly in CCK-8 with some larger forms and precursors (Cantor 1986a).

All these forms share a common C-terminal structure, similar to the C-terminal end of gastrin, which will determine biological activity and receptor affinity (Table 2). Sulphatation of the tyrosine residue and amidation of the C-terminal part are essential for full stimulating activity of CCK (Jensen 1981). Sulphated CCK is 100–300 fold more potent than non-sulphated CCK and non amidated forms have no activity at all (Eipper 1985). Differences in tertiary structure of the carboxyl terminus of CCK may influence receptor binding, stability to enzymatic digestion and biological effects (Reeve 2002).

Table 2

AMINO ACID SEQUENCE OF CCK MOLECULAR FORMS

Preprocholecystokinin

MNSGVCLCVLMAVLAAGALTPVPPADPAGSGLQRAEEAPRRQLRVVSQRTDGE**SR**AHLGALLA**RY**IQQARKAPSGRMSIV**KN**LQNLDPSHRISD**RD**YMGWMD**DF**GRRSAAEEYEYPS

	CCK-83	CCK-58	CCK-39	CCK-33	CCK-22	CCK-8	NH ₂
CCK-83	Gln-Pro-Val-Pro-Pro-Ala-Asp-Pro-Ala-Gly-Ser-Gly-Leu-Arg-Ala-Glu-Glu-Ala-Pro-Arg-Arg-Gln-Leu-Arg-Val-Ser-Gln-Arg-Thr-Asp-Gly-Glu-Ser-Arg-Ala-His-Leu-Gly-Ala-Leu-Leu-Ala-Arg-Tyr-Ile-Gln-Gln-Ala-Arg-Lys-Ala-Pro-Ser-Gly-Arg-Met-Ser-Ile-Val-Lys-Asn-Leu-Gln-Asn-Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr(SO ₃ ⁻)-Met-Gly-Trp-Met-Asp-PheNH ₂						
CCK-58	Val-Ser-Gln-Arg-Thr-Asp-Gly-Glu-Ser-Arg-Ala-His-Leu-Gly-Ala-Leu-Leu-Ala-Arg-Tyr-Ile-Gln-Gln-Ala-Arg-Lys-Ala-Pro-Ser-Gly-Arg-Met-Ser-Ile-Val-Lys-Asn-Leu-Gln-Asn-Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr(SO ₃ ⁻)-Met-Gly-Trp-Met-Asp-PheNH ₂						
CCK-39	Tyr-Ile-Gln-Gln-Ala-Arg-Lys-Ala-Pro-Ser-Gly-Arg-Met-Ser-Ile-Val-Lys-Asn-Leu-Gln-Asn-Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr(SO ₃ ⁻)-Met-Gly-Trp-Met-Asp-PheNH ₂						
CCK-33	Lys-Ala-Pro-Ser-Gly-Arg-Met-Ser-Ile-Val-Lys-Asn-Leu-Gln-Asn-Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr(SO ₃ ⁻)-Met-Gly-Trp-Met-Asp-PheNH ₂						
CCK-22	Asn-Leu-Gln-Asn-Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr(SO ₃ ⁻)-Met-Gly-Trp-Met-Asp-PheNH ₂						
CCK-8	Asp-Tyr(SO ₃ ⁻)-Met-Gly-Trp-Met-Asp-PheNH ₂						
CCK-4	Trp-Met-Asp-PheNH ₂						
Cerulein	pGlu-Gln-Asp-Tyr(SO ₃ ⁻)-Met-Gly-Trp-Met-Asp-PheNH ₂						
Cionin	pGlu-Gln-Asp-Tyr(SO ₃ ⁻)-Met-Gly-Trp-Met-Asp-PheNH ₂						
Gastrin-17	Tyr-Gly-Pro-Trp-Leu-(Glu)S-Asp-Ala-Tyr-Gly-Trp-Met-Asp-PheNH ₂						

Legend: The sequence in italic represents the C-terminal heptapeptide amide sequence that is the minimal epitope necessary for receptor binding shared by all active CCK forms, the underlined pentapeptide amide sequence is common to gastrin and CCK molecular forms, amino acids in bold italic show the cleavage monobasic sites, the circled amino acids are the site of amidation to release bioactive carboxyamidated CCK peptides.

3-Biosynthesis and processing

The CCK complementary DNA has been cloned in several species, including man (Takahashi 1985) and rat (Deschenes 1985). The same gene transcript of 534 bp is expressed in the intestine and in the nervous system (**Figure 1**). The 345 mRNA bases code for a 115-amino-acid prepropeptide composed of a 20-amino-acid signal peptide, a 25-amino-acid linking segment, CCK-58, and a 12-amino-acid carboxyterminal extension (Takahashi 1985). Significant progress has been made in the elucidation of the mechanism and enzymology of pro-CCK cleavage using endocrine tumour cells which process pro-CCK

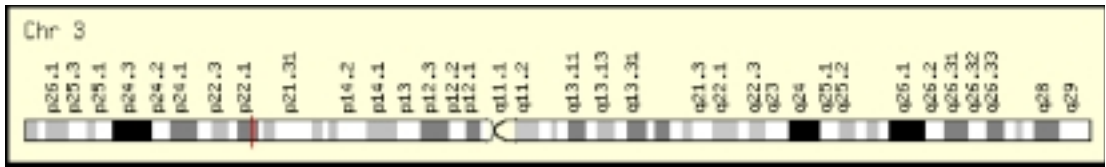
to CCK 8 (Beinfeld 1992). CCK biosynthesis takes place in the context of the regulated secretory pathway. After insertion into the endoplasmic reticulum and removal of the signal peptide, pro-CCK assumes its correct, three-dimensional structure. From there it is probably not further modified until it reaches the trans-Golgi. In the trans-Golgi, three out of the four tyrosine residues of pro-CCK are sulphated by a specific membrane-bound tyrosine sulphotransferase (Niehrs 1990). The sulphatation of one of the tyrosine residues (in CCK-8) is important for its biological activity at CCK-1 receptors. Recent evidence suggests that sulphatation of the tyrosine in pro-CCK allows for correct sorting and/or processing of pro-CCK in an endocrine cell in culture (Beinfeld 1994).

In the trans-Golgi network, sulphated pro-CCK is sorted into secretory granules with other material destined for the regulated secretory pathway. The struc-

Figure 1

CCK GENE LOCATION AND STRUCTURE

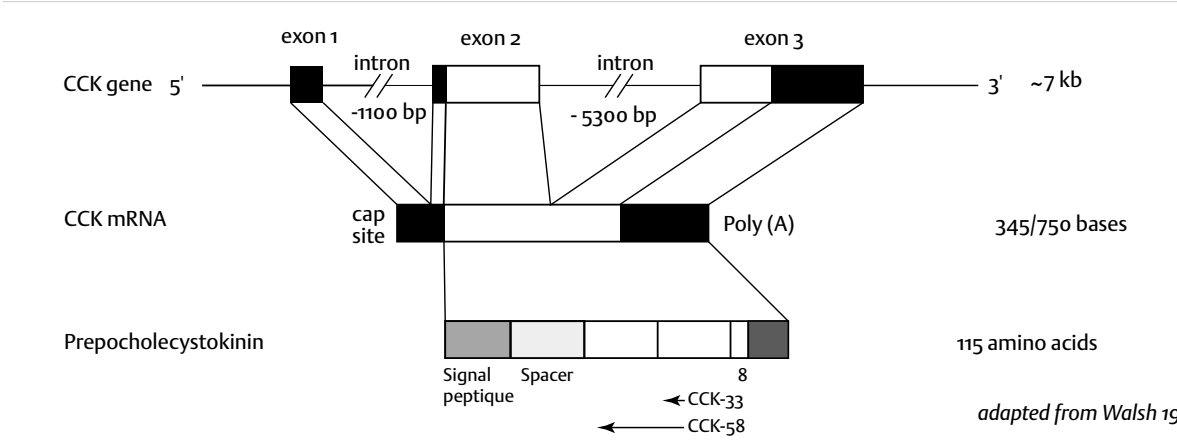
Location of CCK gene: 3p22-p21.3



CCK GENE SEQUENCE 534 BP

ttttccatt gctctccttc ccaacaaatt gaactagaga tgcttttaga tgcaatgtcc	60
ctgttcctt cctccctgct ccttgtagct ccttctggac gaatgtccat cgtaagaac	120
ctgcagaacc tggacccag ccacaggata agtgaccggg actacatggg ctggatggat	180
tttgccgtc gcagtgccga ggagtatgag taccctctct agaggacca gccgccatca	240
gccaacgga agcaacctcc caaccagag gaggcagaat aagacaaca tcacactcat	300
aactcattgt ctgtggagtt tgacattgaa tgatatctatt tattaagttc tcaatgtgaa	360
aattgtgtct gtaagattgt ccagtgaac cacacacgct caccagaagt tgtgcaaact	420
gaagacaaaa ctgtttctt catctgtgac tctgttctg aaaatgttgt tatgtatta	480
aagtgatttc attctgccgt gtcgttcct gcgtctactg agggcaaagg gctc	534

CCK GENE STRUCTURE AND PROCESSING TO PREPROCHOLECYSTOKININ



tural features of pro-CCK (or any proneuropeptide) that allow it to be recognized as secretory material are unknown. A membrane-bound form of carboxypeptidase E has been proposed as a sorting receptor for neuropeptide prohormones (Cool 1997).

The most probable model of the processing of pro-CCK to CCK-8 involves the following steps: 1) signal peptide cleavage (by signalase in endoplasmic reticulum membrane); 2) cleavage of pro-CCK at a single arginine site to release carboxyl terminal extended CCK-8; 3) removal of the carboxyl-terminal extension by cleavage at an ArgArg site; 4) the possible action of a carboxypeptidase E activity to remove extra arginines on the carboxyl terminal to produce CCK-8Gly; 5) conversion of CCK-8Gly to CCK-8 amide by the amidating enzyme (Eberlein, G. A. 1992). The processing of pro-CCK is somewhat unusual because most of the cleavage sites occur at single Arg residues. This is not true for pro-gastrin, although many other features of the prohormone structure have been retained in evolution.

The pathways for posttranslational processing may differ between the gut and the nervous system, but there is some evidence for similar processing *in vivo*, which may seem to differ between organs because of *in vitro* differences in degradation in smaller forms (Rehfeld 1986, Reeve 1991). The enzyme(s) responsible for the degradation of CCK-8 after it is released has not been definitely identified, but a likely candidate (a serine peptidase) has recently been isolated and cloned (Rose 1996).

4-Cellular/molecular mechanisms regulating CCK cell differentiation

The four epithelial cell types of the intestine, enterocytes, goblet cells, Paneth cells and enteroendocrine cells originate from a common totipotent stem cell in the intestinal crypts. The intestinal epithelium of mammals is perpetually self-renewing and the cells turnover every 3-5 days (Cheng 1974). Stem cell descendants then give rise to progenitors for each of the major cell lineages. Cells differentiate as they migrate up the crypt-villus axis. As cells enter the villus compartment during differentiation they stop dividing, eventually undergo apoptosis and are extruded into the intestinal lumen.

Relatively little is known about transcriptional events that direct differentiation of the intestinal epithelium. Until recently, the efforts to apprehend these mechanisms have been hampered by the paucity of isolated genes and of well-differentiated cell

lines that reflect the properties of differentiated enterocytes, goblet cells, enteroendocrine cells and Paneth cells. Concerning enteroendocrine cells, at least ten different types have been described in the small intestine of mammals (Solcia 1998). Several cell types may co-express more than one hormone (Fiasse 2001). The heterogeneity of hormone expression in enteroendocrine cells is believed to reflect their peculiar multipotency and is also observed in gut neuroendocrine tumours and derived cell lines (Madsen 1986).

Several characteristics of secretin cells made them an attractive model for the study of intestinal differentiation. These studies demonstrate the existence of a multipotent endocrine-committed cell type and locate this hybrid multipotent cell type to a region of the intestine populated by relatively immature cells (Rindi 1999). This early progenitor cell does not express the hormone gene and differentiates into late intermediate cells expressing the secretin gene. At least three terminally differentiated enteroendocrine lineages including CCK cells, secretin cells and glucagons/peptide YY cells segregate from this late progenitor cell. Other cell lineages expressing gastrin, somatostatin and other hormones may differentiate through other progenitors or pathways. It is however still unclear if each cell type differentiates from the multipotent progenitor on the basis of a predetermined differentiation cascade or in response to specific physiological stimuli. As these cells migrate up the crypt-villus axis, they produce secretin and stop dividing as they terminally differentiate and die. Transcription of the secretin gene is controlled by a complex enhancer binding to multiple transcription factors. Studies both in cell lines and in knockout mice highlight the importance of the basic helix-loop-helix protein (bHLH), BETA2, for expression of the secretin gene. BETA2, a tissue-specific bHLH protein expressed in the pancreas, intestine and brain, was cloned from a pancreatic islet cell line and identified as an activator of the insulin gene. BETA2 knockout mice failed to develop secretin- and cholecystokinin-expressing enteroendocrine cells, whereas the other enteroendocrine lineages were relatively unaffected by the absence of a functional BETA2 gene. The close relationship between secretin and CCK-expressing enteroendocrine cells was also highlighted by this model (Naya 1997).

In addition, BETA2 may occupy a key position in coordinating cell cycle arrest with secretin gene expression in terminally differentiating secretin cells. The ongoing renewal of intestinal epithelium must be balanced by turnover of existing cells. The ability of BETA2 to induce apoptosis may indicate a role for this protein in cell number regulation, and represents the first example of transcriptional control of this process in the intestinal tract. Regulation of secretin cell number by BETA2 may also represent a mechanism for limiting the size of a relatively non-abundant sub-

population of differentiated cells with respect to other epithelial cell lineages. Thus, BETA2 may be involved in coordinating the entire cycle of differentiation cells from secretin expression through cell cycle arrest to cell turnover. As such, it may represent the first example of a master regulatory gene controlling intestinal differentiation (Mutoh 2000). Other transcription factors have been suggested to be involved in this process (Yamada 2001).

5-Cellular/molecular mechanisms regulating CCK gene expression

5.1 CCK gene and promoter

The CCK complementary DNA has been cloned in several species, including human (Takahashi 1985) and rat (Deschenes 1985). Despite the physiological relevance of the peptide, the factors involved in the regulation of CCK gene transcription are little known. The first studies concerned the transcriptional expression of CCK in nervous tissue, especially in neuroblastoma cell lines (Hansen 1999). Cloning and characterization of the CCK promoter region has identified several recognized regulatory DNA sequences, including consensus binding sites for a number of transcription factors, within 100 bp of the transcriptional start site (Haun 1990). This proximal upstream regulatory region of the CCK gene exhibits functional elements highly conserved in evolution. Of particular interest is a putative composite calcium response element (CRE)/activator protein 1 (AP1) site that may bind both the calcium/3,5'-monophosphate response element binding protein (CREB) and AP1 (Haun 1990). Composite CRE/AP-1 sites of a similar type occur in a number of genes involved in neurotransmitter synthesis, including dopamine β -hydroxylase, prodynorphin, and proenkephalin (Shaskus 1992). CRE/AP1 sites are potentially able to bind members of both the CREB/activating transcription factor and AP1 transcription factor families, thereby facilitating transcriptional integration by a wide range of stimuli, representing convergence of separate signal transduction pathways (Nielsen 1996).

The CCK promoter exhibits increased activation in response to overexpression of CREB and the AP1 dimerization partners, Fos and Jun, in a neuronal cell line. Promoter activation with basic fibroblast growth factor, via activation of CREB, has also been demonstrated in the neuronal cell line. This has been proposed as a mechanism by which growth and neu-

rotrophic factors, in concert with neuropeptides, may regulate expression of the CCK gene (Haun 1990). The CRE/AP1 site in the CCK promoter lies downstream of an E box binding domain for the basic helix-loop-helix family of transcription factors, and interaction between these two sites in cAMP-stimulated activity has recently been described (Rourke 1999).

5.2 CCK gene expression and nutrients

That the functional elements identified in nervous tissue are operating as well in the enteroendocrine cells is likely but not proven. Agents stimulating CCK-storing sites clearly differ in the central nervous system and in the gut. Indeed, intestinal endocrine cells are polarized epithelial cells, with their apical pole in direct contact with the intestinal lumen and with the alimentary chyme. Theoretically, endocrine cells in the mucosa could respond to luminal nutrients directly through membrane structures or indirectly through nervous or paracrine intermediates (Buchan 1999). There is evidence from studies *in vivo* that dietary and hormonal factors influence intestinal CCK mRNA abundance (Liddle 1994). Food intake is the most potent stimulant of CCK release and there is a correlation between diet-induced CCK gene expression and peptide secretion in rat (Liddle 1988) and men (Bentouimou 1995). Infusion of soybean trypsin inhibitor into the duodenum of anesthetized rats for 12–24 h induced a 3- to 4-fold increase in the CCK RNA level that was the result of an enhanced transcriptional activity of the CCK gene (Liddle 1988, Playford 1993). The rationale of the experiment, however, was stimulation of CCK release through interruption of the negative feedback with luminal trypsin, not the effect of peptones. In man, ingestion of mixed nutrients was followed by an increase in the CCK messenger RNA level in extracts of mucosal biopsies (Bentouimou 1995). Nevertheless, these studies were based on *in vivo* dietary manipulations and, for that reason, did not permit identification of the molecular and cellular mechanisms involved.

Few data are available on the signaling pathways connecting dietary changes to alterations in gene expression. Most of the current knowledge concerns the effects of fatty acids and glucose in the transcriptional control of genes encoding proteins that play significant roles in lipid and glucose transport or metabolism in hepatocytes, adipocytes, or pancreatic beta cells (Duplus 2000, Vaulont 2000). The mechanisms involved were elucidated in some cases, and DNA response elements, binding transcription factors, and transduction cascades were identified. For instance, a large part of the transcriptional effects of fatty acid can be assigned to the direct activation of peroxisome proliferator-activated receptors (Forman 1997). The transcriptional effect of glucose has been shown to be mediated by an AMP-activated protein

kinase pathway or by a phosphatidylinositol 3-kinase pathway (Macfarlane 1999, da Silva Xavier 2000). Much less is known about the effect of protein metabolites, but an amino acid response element that bound the activating transcription factor 2 has been shown to induce promoter activity in cells of different origins (Bruhat 2000).

5.3 CCK gene expression in STC-1 cell line

Factors controlling CCK release have been relatively well studied both *in vivo* and in cell lines such as STC-1 (Liddle 1986, Snow 1994, Chang 1996, McLaughlin 1998). The STC-1 cell line is derived from an intestinal endocrine tumour, established from an endocrine tumour of the small bowel that developed in a double-transgenic mouse expressing the rat insulin promoter linked to the SV40 large T antigen and the polyoma small t antigen. These cells express CCK mRNA and secrete the biologically active form of the peptide, CCK-8, thus providing a useful model of the CCK-producing endocrine cell (Rindi 1990). In STC-1 cells, CCK is released by amino acids, peptones and fatty acids as well as by secretagogues such as bombesin and PACAP (Snow 1994, Chang 1996, McLaughlin 1998, Nemoz-Gaillard 1998b).

Digestion of proteins mainly yields amino acids and oligopeptides. Protein hydrolysates (i.e. peptones) not only stimulate CCK secretion *in vivo* but also *in vivo* in the model of isolated vascularly perfused rat intestine (Cuber 1990) and *in vitro* in the intestinal enteroendocrine cell line STC-1 (Cordier-Bussat 1997). The *in vitro* model allowed to identify, in the CCK gene promoter, a DNA cis-element that was required for the transcriptional response to protein hydrolysates (Bernard 2001). The effect of protein hydrolysates on STC-1 cells is in accordance with the hypothesis of a direct effect of nutrients on intestinal endocrine cells *in vivo* (Cordier-Bussat 1997). Furthermore, it should be noted that CCK mRNA level was not affected by protein hydrolysates in other CCK-expressing cell lines from various origins, suggesting an intestinal-specific nutrient-dependent mechanism in enteroendocrine cells such as STC-1 cells (Cordier-Bussat 1997). Among the four CCK-expressing cell lines tested, only the STC-1 cell line, displayed a response pattern that would fit the alleged reactivity of intestinal I cells to luminal nutrients.

5.4 Neuropeptides and PACAP

In vivo, CCK is not only stimulated by nutrients, but also by neuropeptides including pituitary adenylate cyclase-activating polypeptide (PACAP), bombesin, and luminal CCK releasing peptides such as monitor

peptide and the luminal CCK-releasing factor LCRF. The mechanisms by which neuroendocrine stimulants regulate CCK gene transcription are unclear. PACAP is a member of the secretin/glucagon/vasoactive intestinal polypeptide (VIP) regulatory peptide family and was originally identified in the hypothalamus as a stimulant of pituitary adenylate cyclase (Miyata 1992). This neuropeptide is expressed *in vivo* by both central and peripheral neurons, including those of the submucosal plexus underlying the enteroendocrine cells. PACAP has been shown to stimulate CCK secretion from the STC-1 cell line and from a mucosal I cell-enriched preparation from rat small intestine (Chang 1996) and has therefore been proposed as a candidate neuromodulator of CCK release *in vivo* (Lee 1998). The mechanisms behind such secretagogue effects are known to be associated with rises in intracellular calcium and cAMP levels and to act through at least three receptor subtypes (Chang 1996, Harmar 1998). PACAP-induced CCK gene expression was recently demonstrated to be achieved by CREB family transcription factors acting at the CRE/AP1 site (Deavall 2000). PACAP increases CCK gene expression via a cAMP-mediated pathway involving CREB phosphorylation by protein kinase A and activation of this composite CRE/AP1 site.

6-CCK-1 and CCK-2 receptors

A vast literature exists on the pharmacology and physiology of CCK. By the early 1980s, it was clear that two major sub-types of CCK receptors existed. Both of these have equally high affinity for CCK-8 sulphate, while they differ substantially for non-sulphated CCK peptides, gastrin and amidated peptides shorter than CCK-7, like CCK-4 and CCK-5 (also called pentagastrin). The CCK-1 receptor subtype (previously CCK-A, A for alimentary), typically found in the pancreas, is relatively specific for sulphated CCK-8. Non-sulphated CCK-8, CCK-4 and gastrin are 2–3 orders of magnitude less potent (Jensen 1980). For the CCK-2 (previously CCK-B, B for brain) receptor subtype, the difference in potency between sulphated CCK-8, non-sulphated CCK-8, gastrin and CCK-4 is about one order of magnitude. In April 1992, in the same issue of the Proceedings of the National Academy of Science, the cloning of the rat pancreatic CCK-1 receptor (Wank 1992) and the canine parietal gastrin receptor were reported (Kopin 1992). Both appear to be classical seven-transmembrane domain proteins of about 450 amino acids that are 48% identical to each other (Pisegna 1992). Unlike other neurotransmitter receptor systems (e.g., acetylcholine, serotonin and somatostatin), the CCK system, with only two receptor subtypes, appears to be a model of simplicity.

The CCK-1 receptor can be found in the pancreatic acini, the gastric mucosa, gallbladder, intestinal smooth muscle, and in various cancer cell lines. This type of receptor was also found in the brain in a surprisingly wide range of regions, specifically in the interpeduncular nucleus, area postrema and the nucleus tractus solitarius (Moran 1986, Hill 1987) and seems to mediate nociception in primates (Hill 1988a, Hill 1988b). Respective alimentary and brain role of CCK and its receptors will be described in the following sections.

The CCK-2 receptor is more prevalent in the brain than CCK-1 and less in the periphery neurons. These receptors are widely distributed in the brain, especially in the limbic regions associated with emotions (amygdale, hippocampus), cortex, and ventromedial hypothalamus (Woodruff et al., 1991). The functions of this receptor will be further developed in the CCK brain section below. Subsequently, it was found that the gastrin receptor in the stomach was the same as the CCK-2 receptor found in brain (Saito 1980). In the stomach, it plays an important role in mediating gastric acid secretion and mucosal growth (Langhans 1997).

The mechanisms of stimulation of CCK receptors by their respective ligands have been fully studied on pancreatic acini cells, where CCK stimulates release of digestive enzymes. Binding of CCK to its receptors activates protein G and coupling to phospholipase C which leads to inositol biphosphate hydrolysis into inositol 1,4,5-triphosphate (IP₃) and 1,2-diacylglycerol (DAG) (Shulkes 1997). The increase in IP₃ levels mobilizes intracellular calcium and DAG activates protein kinase C (PKC), which delocalises from the cytosol to the membrane. Activation of intracellular kinases leads to multiple protein phosphorylation and than to digestive enzyme secretion (Wank 1995).

Another receptor has been described on a colon tumour cell line (HCT116) and in the gastric mucosa. It has a weak affinity for gastrin and CCK and seems to be the target for the antiproliferative effects of CCK-receptor antagonists (Baldwin 1994). Different receptors were shown on the LoVo colon cell line (Bold 1994) and in pig pancreatic islets capable to stimulate insulin, glucagon, somatostatin and pancreatic polypeptide release (Rehfeld 1980).

7-CCK receptor antagonists

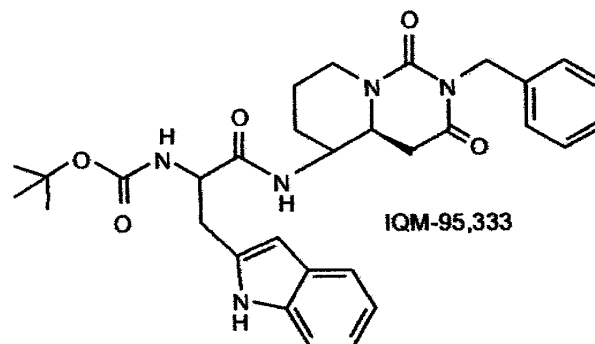
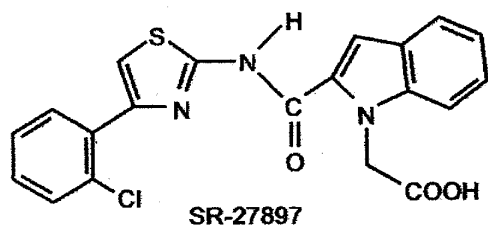
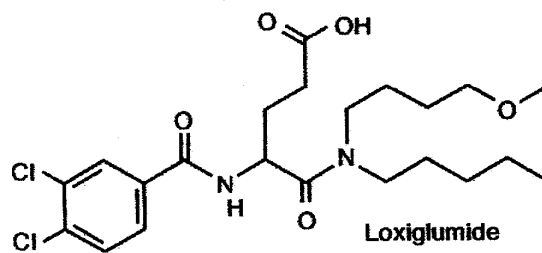
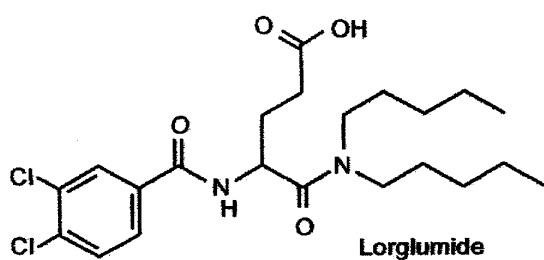
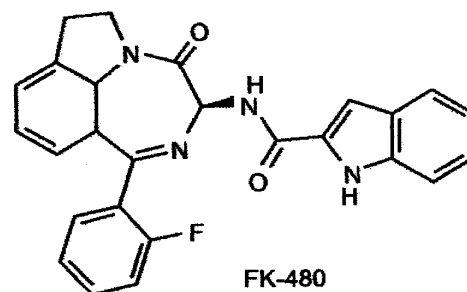
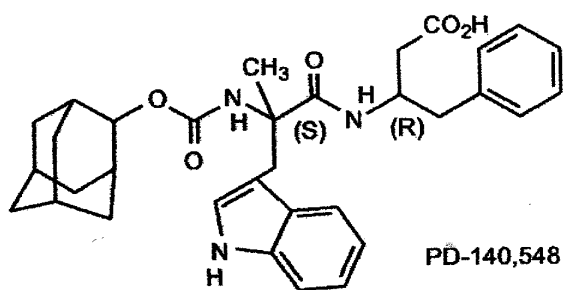
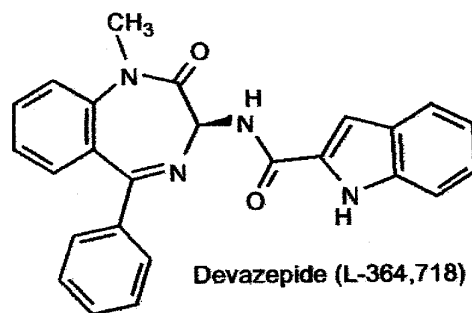
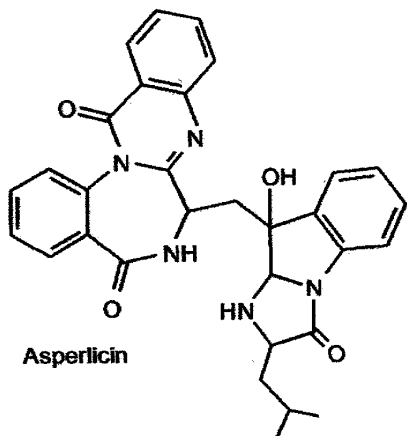
Different classes of receptor antagonists have been developed to provide further understanding in CCK biological functions. Some of these have been used extensively *in vitro* to characterize CCK receptors and have been used *in vivo* to study physiological effects of CCK. They are now used in several clinical trials and their therapeutic potential will be discussed in

the chapters about digestive and brain functions of CCK. These CCK receptor antagonists include (**table 3 and table 4**):

- cyclic nucleotide derivatives such as dibutyryl cGMP (Peikin 1979)
- derivatives of glutaramic acid such as:
 - ° proglumide and benzotrypt (Hahne 1981), which now has been superseded by A65186 (Vickroy 1988)
 - ° lorglumide (CR-1409) and the less toxic loxiglumide (CR-1505), both highly specific for CCK-1 receptors (Setnikar 1988)
 - ° dexloxiglumide, the (R)-isomer of loxiglumide, is a new selective and highly potent CCK-1 receptor antagonist (Varga 2002)
- CCK peptides altered at the carboxyl terminus such as:
 - ° BOC-[D-Trp₃₀,Nle₃₁]CCK/27-32)-phenyl ethyl ester (Lignon 1987)
 - ° CCK-8 derivatives in which aminohexanoic acid was substituted for methionine, BC 254 (Charpentier 1988)
 - ° CCK-4 restricted analogs with a 3-oxoin-dolizidinering, IQM-95,333 (Martin-Martinez 1997).
- derivatives from a naturally occurring benzodiazepine, aspercilin, which has been isolated from the fungus *Aspergillus alliaceus*:
 - ° devazepide (also known as MK-329 or L-364,718), which remained for several years the most potent and selective antagonist for CCK-1 receptors (Lotti 1987)
 - ° its analogue, L-365,520, which has a high potency and selectivity for CCK-2 receptors in rats (Lotti 1989)
 - ° other selective compounds are: L-740,093 (Patel 1994), YMO22 (Nishida 1994), YF476 (Takinami 1997), which have potent CCK-2 receptor affinity
 - ° recently, another potent CCK-1 receptor antagonist FK-480 (Ito 1994).
- more recently other non-peptidic antagonists have been developed:
 - ° dipeptoids with tryptophan derivatives such as PD-134,308 (CI-988) and PD-135,158 (Hughes 1990)
 - ° diphenylpyrazolidones, LY-262,691 (Rasmussen 1991)
 - ° ureidoacetamides, RP-73,870 (Pendley 1995)
 - ° various: JB-93,182 and AG-041R, CR-2194 and CR-2945 (Makovec 1999) all of them with a high affinity for the CCK-2 receptor (Hakanson 1999) and SR-27,897 with a high affinity for the CCK-1 receptor (Gully 1993).
- several other dipeptoids were synthesized, as:
 - ° 2-NAP [2-naphtalenesulfonyl 1-aspartyl-(2-phenethyl)amide] (Hull 1993)
 - ° PD-140,548 (Boden 1993) with a potent and selective antagonist effect for CCK-1 receptors.

Table 3

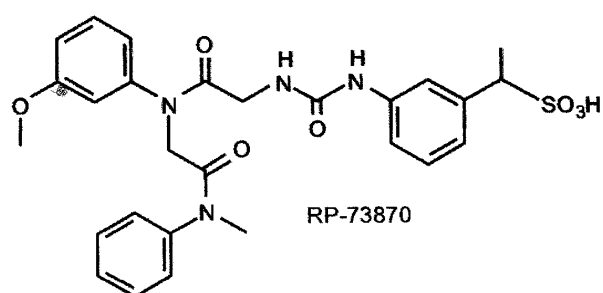
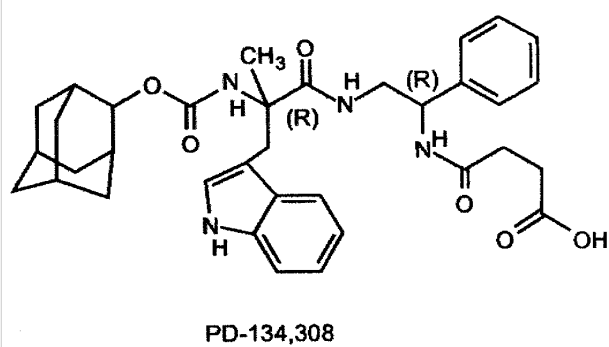
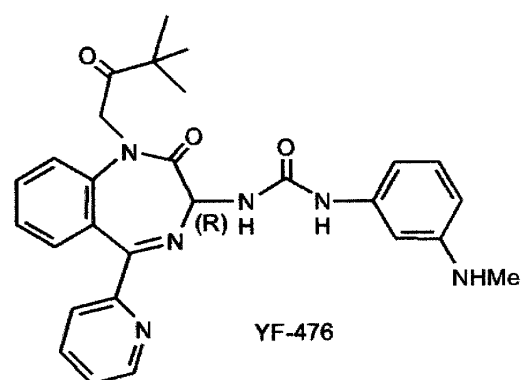
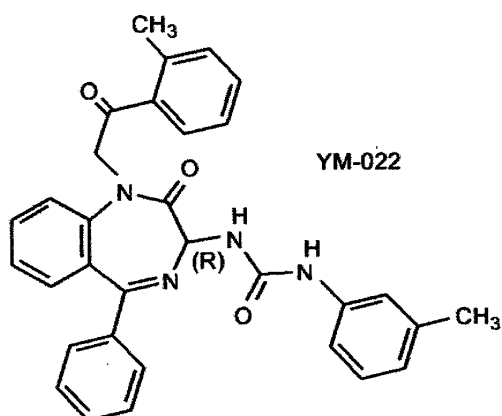
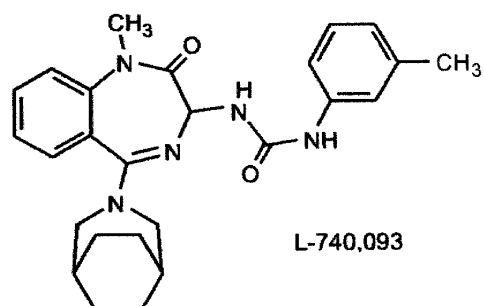
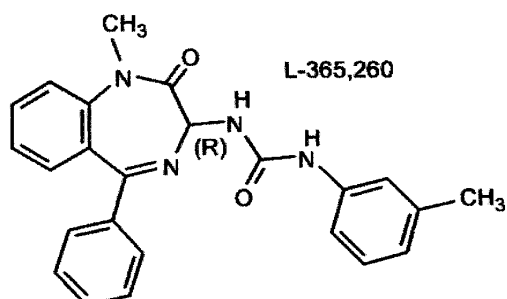
CCK-1 RECEPTOR ANTAGONISTS



adapted from Noble 2002.

Table 4

CCK-2 RECEPTOR ANTAGONISTS



adapted from Noble 2002.

8-Regulation of secretion

8.1 Effects of nutrients on CCK secretion

Plasma CCK concentrations measured in the circulation by equally sensitive and specific radioimmunoassays and bioassays average 1pM in the fasting state and 5-10 pM postprandially. Bioassay studies showed that the food components capable of stimulating pancreatic secretion most actively in men were fat, fatty acids (oleate), protein and amino acids (e.g. tryptophan and phenylalanine) (**Table 5**). Intraduodenal administration of saline, dextrose and glycerol did not affect pancreatic secretion. The stimulatory effect of fat is only observed after intraluminal administration and intravenous infusion of fatty acids does not affect CCK plasma levels (de Boer 1992).

Concerning lipids, it was shown that long-chain triglycerides are potent stimuli, whereas medium-chain triglycerides are not (Hopman 1984b, Schaffalitzky de Muckadell 1986, Isaacs 1987). Triglycerides require hydrolysis and dispersion of fatty acids into micelles to become effective stimulants. Fatty acids with a chain of 12 carbon atoms or longer are strong stimulants of CCK secretion, whereas those of 11 or fewer carbon atoms failed to increase plasma CCK. A highly specific, chain length-sensitive fatty acid recognition system does seem to exist in the proximal gut mediating CCK secretion and gastric motility (McLaughlin 1999). Unsaturated fatty acids (such as oleic acid) evoke a much greater release of CCK than saturated fatty acids (stearic acid) (Beardshall 1989). Apart from lipolysis, fatty acid chain length, and saturation, emulsification may also determine the magnitude of CCK release (Ledeboer 1999). The observation that free fatty acids of chain length C12 and above release CCK *in vivo* suggests

that chylomicron formation may be involved, since these are the fatty acids that are absorbed via chylomicron formation. The role of chylomicron formation on release of CCK *in vivo* was studied by a perfusion of an emulsion of long-chain triglycerides in the rat that rapidly increased the plasma level of CCK (Raybould 1998).

Concerning proteins, no major differences were shown between elemental (amino acid) and polymeric (protein) meals in healthy volunteers (Fried 1989, Deprez 2002b). Of the individual amino acids, tryptophan and phenylalanine appear the most potent stimulants in humans (Go 1970). In man, the effect of parenteral amino acids on CCK release is disputable (Nealon 1990). Administration of starch does not affect plasma CCK concentrations (Hopman 1985).

The relationship between diet, cholecystokinin and pancreatic growth and carcinogenesis has gained interest since rats fed with raw soya flour were shown to develop enlarged pancreas, preneoplastic lesions and carcinomas in their pancreas (Melmed 1969, Roebuck 1987a, Watanapa 1992a, Watanapa 1992b, c, 1993a). This effect was shown to be mediated by heat sensitive soybean trypsin inhibitors (Temler 1986). Fatty acids also enhance pancreatic carcinogenesis, whereas protein appears to have a protective role. It was however shown that certain plant proteins enhance the growth of carcinogen-induced pancreatic foci and that this effect is considerably greater than the enhancement by high levels of dietary unsaturated fat (Roebuck 1987b). Diets high in trypsin inhibitor were also shown to stimulate CCK release in men (Calam 1987). Moreover lectins, such as soybean lectin, appeared to be strong stimulants of pancreatic secretion and CCK release, a mechanism mediated through CCK₁ receptors (Deprez 1991, Deprez 1992, Jordinson 1996, Jordinson 1997). CCK is the key intermediary in these response, since CCK antagonists largely prevent these changes (Deprez 1992, Playford 1993).

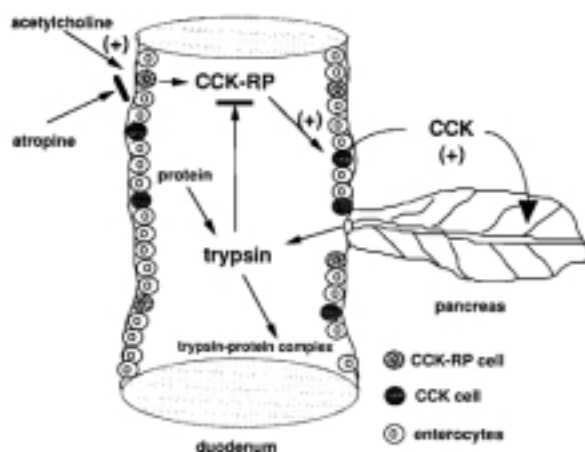
Table 5

INTESTINAL CCK RELEASE REGULATION BY NUTRIENTS

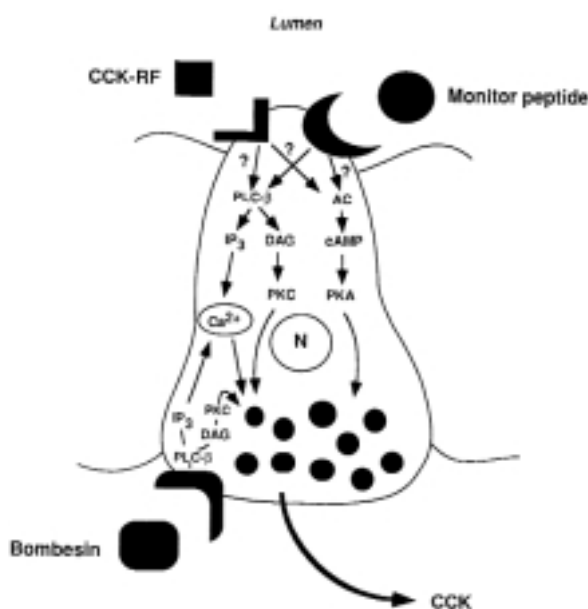
Stimulate CCK release	Potentiate CCK release	Inhibit CCK release
Protein/Amino acids/Peptones	Bile acid binding resins (cholestyramine)	Bile acids
Fat/ Tryglicerides/ Fatty acids >12 C	Protease inhibitors	Pancreatic enzymes
(Glucose)	Ethanol	

Adapted from Liddle 2000

Figure 2



Panel A: Model of mechanism by which a CCK-releasing peptide (CCK-RP: monitor peptide or LCRF) could stimulate the secretion of CCK postprandially. CCK-RP is secreted by specific intestinal cells into the gut lumen where it gets inactivated by trypsin. When food enters the duodenum, ingested proteins bind to trypsin and prevent CCK-RP from being inactivated. This model is the basis of the "feed-back" regulation of CCK by the enzymes produced in the pancreas following CCK stimulation. CCK-RP could also stimulate CCK release directly by a specific receptor on the CCK cell (B).



Panel B: Model for intracellular signaling in the CCK cell. Evidence that both adenyl cyclase and phosphoinositide cascades exist in CCK cells makes it possible that potential secretagogues such as bombesin and CCK-RF bind to cell surface receptors and initiate a series of signaling events to ultimately cause CCK secretion.

Adapted from Liddle 1995 and Herzig 1996

8.2 Feedback regulation of CCK secretion

8.2.a Feedback regulation in rats

Pancreatic proteases in the proximal small intestinal lumen exert a negative feedback control on pancreatic enzyme secretion in several species, including man (Green 1972, Ihse 1977, Slaff 1984, Liener 1988, Layer 1990). In rats there is evidence that CCK mediates this feedback mechanism. Firstly, exclusion of bile and pancreatic juice is a most potent and long-lasting stimulation of CCK release and pancreatic secretion (Green 1972, Miyasaka 1984, Owyang 1986a, Owyang 1986b). Secondly, intraluminal proteins release plasma CCK, and this CCK response is abolished by simultaneous intraduodenal perfusion with trypsin (Louie 1986, Folsch 1987). Thirdly, the inhibition of the intraduodenal trypsin activity by soybean trypsin inhibitor or the synthetic protease inhibitor camostat increases the release of CCK (Liddle 1984, Goke 1986). According to these findings, rats with exocrine pancreatic insufficiency, in which the concentration of duodenal proteases is fairly diminished, develop a significant increase in basal plasma CCK levels (Folsch 1987).

8.2.b CCK releasing peptides

At least three lumenally secreted peptides have been shown to stimulate CCK release when administered intraduodenally. The first one is an endogenously produced CCK-releasing factor secreted into the gut lumen during exclusion of bile and pancreatic juice in rats (Lu 1989, Miyasaka 1989). This factor, known as monitor peptide, has been chemically characterized (Iwai 1987). It is produced by pancreatic acinar cells and secreted by way of the pancreatic duct into the duodenum. On reaching the small intestine, monitor peptide interacts with CCK cells to induce CCK release. Recently, a new CCK-releasing factor, luminal CCK-releasing factor (LCRF), has been discovered from rat intestinal perfusate (Spannagel 1996). This peptide is composed of 70 to 75 amino acid residues and has a molecular mass of 8136 Daltons. LCRF is present in abundance in the duodenal epithelial cells (Tarasova 1997) and its release into the lumen is mediated by intestinal submucosal cholinergic neurons (Miyasaka 1999). Functionally, LCRF appears to mediate feedback regulation of pancreatic secretion and CCK release in response to peptone and lipid stimulation (Miyasaka 1997). LCRF was found to be identical to the porcine diazepam binding inhibitor by peptide sequencing and mass spectrometry analysis (Herzig 1996). It fulfils all the criteria as a physiological regulator of CCK secretion. LCRF has also a direct effect on CCK cells (STC-1 cells) to stimulate hormone secretion (Wang 2002). This represents the first chemical characterization of an enteric peptide functioning as an intraluminal regulator of intestinal hormone release (Owyang 1999, Li 2000) (figure 2).

8.2.c Feedback regulation in men

In humans, however, the existence of a CCK-mediated negative feedback is rather controversial. A first study confirmed the existence of a similar feedback in man, in whom the intraduodenal perfusion of trypsin inhibited the stimulation of CCK release and pancreatic enzyme secretion by phenylalanine, oleic acid and a test meal (Owyang 1986a). Soybean trypsin inhibitors present in soya flours were similarly thought to enhance stimulation of CCK release postprandially by this negative feedback mechanism (Playford 1993). Many other studies failed to confirm these findings: no effect of trypsin on oleate stimulation of plasma CCK release (Olsen 1988), no effect of chymotrypsin on CCK meal stimulated release (Beardshall 1992), no effect of strong inhibition of trypsin and chymotrypsin activity in duodenal juice with the serine protease inhibitor camostat (Adler 1988). These conflicting results were emphasized by the findings of low, normal, and high plasma CCK concentrations in patients with chronic pancreatitis or cystic fibrosis (Funakoshi 1985, Slaff 1985, Cantor 1986b). The existence of such a feedback mechanism is however of great interest in patients with chronic pancreatitis, since some studies showed that pancreatic enzyme supplementation suppresses pancreatic chronic pain (Slaff 1984, Toskes 2001).

The role of bile acids in the feedback regulation of CCK release has been the subject of several investigations. Although supra-physiological amounts of bile and bile salts stimulate pancreatic secretion (Ohlsson 2000), perfusion of the duodenum with much lower concentrations of taurocholate reduced stimulated enzyme output (Koop 1990). It has also been shown that conjugated bile acids such as chenodeoxycholate did stimulate CCK secretion, whereas unconjugated bile acids inhibited CCK release (Ray 1997). Bile diversion and intraduodenal administration of bile acid binding resin cholestyramin induced an increase of stimulated CCK plasma concentrations in dogs, whereas cholestyramin augmented amino acid or meal stimulated CCK levels in man. Addition of chenodeoxycholic acid reversed this effect. This suggested that bile acids are key constituents of bile responsible for the modulation of postprandial CCK release and that CCK-producing cells are under constant suppression by intraduodenal bile acids (Koide 1993). In the fasting state however, intraduodenal conjugated bile salts exerted a negative feedback control on gallbladder emptying without affecting cholecystokinin release or antroduodenal motility (van Ooteghem 2002). The regulatory role of bile salts in the absence of nutrients from the gut is still poorly understood and the role of CCK deserves further studies (Thimister 1998b).

8.2.d Feedback regulation and surgery

In this context of feedback regulation, several surgical operations have been shown to affect the pancreas growth in animal studies. Pancreatobiliary diversion and partial gastrectomy stimulate pancreatic growth and enhance carcinogenesis, probably by means of increased CCK release (Watanapa 1992c). Complete duodenogastric reflux has similar effects on the pancreas but the gut peptide involved is gastrin (Watanapa 1992b). Although massive small bowel resection increases pancreatic growth, the marked reduction in caloric absorption probably explains its failure to enhance carcinogenesis (Watanapa 1992a). In the pancreas, as in the large intestine, hyperplasia appears to precede and predispose to neoplasia (Watanapa 1993b).

8.3 Neural regulation of CCK secretion

Vagotomy alters pancreatic and gallbladder responses to various stimuli. The changes found after vagotomy could be due to altered release of CCK or altered responsiveness to CCK. Radioimmunoassay of CCK suggests the latter explanation because no differences in CCK responses to oleate were detected in dogs after truncal vagotomy despite markedly diminished pancreatic and gallbladder responses (Fried 1983). Also gallbladder and pancreatic responses to CCK appear to involve release of acetylcholine from postganglionic neurons (Takahashi 1991, Soudah 1992). In vagotomized patients, plasma responses to a liquid fat meal were higher than in non-operated patients, possibly because of rapid gastric emptying after vagotomy (Hopman 1984a).

8.4 CCK release in the brain

The release of CCK is relatively easy to study because of its abundance. Most studies have focused on release of CCK from brain slices *in vitro*. Some studies used synaptosomes and, more recently, some push-pull and microdialysis. Like other neuropeptides, the release of CCK can be elicited with potassium stimulation, veratridine, or an electrical field. Unlike the catecholamines, CCK requires a relatively strong stimulus for release (Gysling 1987). Psychotomimetic drugs like phencyclidine inhibit CCK release from striatal slices and infusion of CCK 8 into the rostral nucleus accumbens antagonized EEG and behavioural effects induced by PCP, supporting a role for CCK as an endogenous antipsychotic agent (Popoli 1995).

9-Biological functions of CCK in the alimentary tract

CCK has a large number of biological actions that involve the gut and appear to be physiological (**Table 6**). Physiological actions mean that endogenous or exogenous stimulation of CCK release accounts for the major fraction of the stimulated digestive function, and that these conclusions are supported by data obtained with CCK antagonists. Secreted by intestinal *I cells*, CCK seems to stimulate vagal afferent fibres to modify proximal gastrointestinal function, to stimulate pancreatic enzyme secretion and growth, gallbladder contraction, to stimulate the contractile component of intestinal peristalsis, to inhibit gastric acid secretion and emptying, and to release other hormones (insulin, pancreatic polypeptide).

More recently, CCK and CCK-1 or CCK-2 receptor knockout models have provided additional information on the physiological roles of CCK (Funakoshi 1995, Takiguchi 1997, Lacourse 1999, Takiguchi 2002). These studies show that the lack in CCK, CCK-1 or

CCK-2 receptor does not cause an embryonic lethal phenotype (Lacourse 1999, Takiguchi 2002). Knockout mice are viable and fertile, they have normal food intake and weight gain (Kopin 1999). They are susceptible to gallstone formation but do not have diarrhoea or malabsorption (Lacourse 1999), illustrating that the role of CCK in normal gastrointestinal physiology as well as in clinically relevant gastrointestinal pathological states remains largely unclear and in many aspects controversial.

9.1 Pancreas

9.1.a Pancreatic secretion

There are three primary phases of pancreatic secretion (cephalic, gastric, and intestinal) that are regulated by a combination of central stimuli (sight, smell, taste of food), luminal factors (pH, fats, protein), neural innervation (cholinergic reflexes), and stimulatory hormones (secretin, cholecystokinin). The vagal nerve is the main mediator of the cephalic and the gastric phase. The importance of the autonomic nervous system in the intestinal phase is illustrated

Table 6

BIOLOGICAL FUNCTIONS OF CCK IN THE ALIMENTARY TRACT

		CCK effect	Therapeutic target
Biliary tract	Gallbladder contraction	+	CCK to avoid sludge during fasting, CCK antagonists for biliary pain
	Sphincter of Oddi	-	Diagnosis of sphincter of Oddi dysfunction
Pancreas	Enzyme secretion	+	? CCK-1 receptor antagonists in pancreatitis
	Cell growth	+	? CCK-1 receptor antagonists in tumours
	Regulation of carbohydratemetabolism	+	Controversial results
Stomach	Basal acid secretion	+	
	Somatostatin secretion	+	
	Pepsinogen secretion	+	
	Gastric emptying	-	? CCK antagonists for gastroparesis, functional dyspepsia
Oesophagus	Lower Oesophageal sphincter pressure	-	? CCK-1 antagonists to reduce the TLOS
Small bowel	Transit time	+	? CCK antagonists in IBS
Colon	Motor activity	+	? CCK antagonists in constipation predominant IBS

(+) stimulation, (-) inhibition

by the fact that cholinergic blockade with atropine markedly inhibits the response to intraduodenal stimulation with nutrients (Valenzuela 1986, Borovicka 1997).

In addition, immunocytochemical studies have identified several peptides in the pancreatic nerve cell bodies capable of stimulating pancreatic secretion. These include substance P, bombesin, gastrin-releasing peptide (GRP), peptide histidine isoleucine (PHI), calcitonin gene-related peptide (CGRP), and neotensin. Despite causing pancreatic stimulation, the contribution of these peptides to meal-stimulated pancreatic secretion appears to be minimal and has yet to be fully determined.

Secretin and cholecystokinin remain the primary modulators of meal-induced pancreatic secretion (Alphin 1959, Solomon 1994). Exogenous CCK is a strong stimulant of pancreatic enzyme secretion *in vivo* but a weak stimulant of water and bicarbonate secretion. CCK-8, CCK-33 and CCK-58 are equipotent for stimulating pancreatic exocrine secretion. Recent data demonstrated that CCK-1 receptor mRNA is expressed in the human duodenum and gallbladder but not in the human pancreas (Tang 1996, Nishimori 1999). A recent detailed study showed very low expression of both CCK-1 and CCK-2 receptors in human pancreatic acini (Ji 2001). Conversely, human pancreatic exocrine secretion stimulated by ingestion of a meal is markedly inhibited by administration of CCK-1 receptor antagonists (Hildebrand 1990). The effects of CCK-1 receptor antagonists on human pancreatic exocrine secretion are thus most probably mediated by receptors located in the pancreas or even in the intestine on afferent vagal fibres (Funakoshi 1999).

Available evidence suggests that lipid, specifically long-chain fatty acid, is sensed in the wall of the intestine, releases CCK from enteroendocrine cells, and activates extrinsic vagal afferent nerve terminals. However, direct evidence demonstrating a role for CCK in activating vagal afferent fibres in response to lipid is lacking and can only come from direct recordings of vagal afferent fiber activity. It is also clear from recent studies that the products of chylomicron formation, including apoA-IV, are likely to be involved in this process (Raybould 1998).

9.1.b Pancreatic growth

Pancreatic growth is stimulated by exogenous administration of CCK or by administration of trypsin inhibitors (e.g. soybean trypsin inhibitor) (Folsch 1978). The increases in pancreatic weight, DNA, RNA and protein content can be prevented by CCK-1 receptor antagonists (Niedermaier 1987, Wisner 1988). Repeated injections of CCK may also increase the islet cell hormone content (Yamada 1980). The role of CCK in maintaining normal pancreatic growth responses to a normal diet is less well defined.

Studies with CCK receptors antagonists gave conflicting results (Green 1992). Data from mice lacking CCK or the CCK-1 receptor showed that CCK was not essential for pancreatic growth (Lacourse 1999, Takiguchi 2002). In the CCK-1 receptor deficient Otsuka Long Evans Tokushima Fatty (OLETF) rats, the pancreatic wet weight was however low and the pancreatic growth after weaning was retarded (Miyasaka 1996). These differences can be due to species variances or to other genetic abnormalities suggested in OLETF rats (Watanabe 1999).

9.1.c Regulation of carbohydrate metabolism

Recently, OLETF rats with disruption of the CCK-1 gene were shown to develop glucose intolerance associated with aging (Funakoshi 1995, Takiguchi 1997). It was known that exogenously administered CCK could enhance insulin release in several species (Szecowka 1982, Mossner 1992). In men the levels of CCK required to enhance amino acid-stimulated insulin release are in the physiological ranges (Rushakoff 1987). Although CCK appears to regulate insulin release in rats, specific CCK-1 receptor antagonism failed to modify insulin responses to meals in men (Niedermaier 1992b). In recent studies, lorglumide blocked gallbladder emptying, accelerated gastric emptying, abolished all phases of the postprandial pancreatic polypeptide response and increased insulin secretion without alteration of glucose profiles (Hidalgo 2002).

9.1.d Clinical impact in pancreatic diseases

The role of CCK antagonists is under evaluation in patients presenting with acute and chronic pancreatitis. The evidence for a role of CCK in induction and development of acute pancreatitis includes the following: first, intravenous administration of large doses of CCK analogues in rats and mice can cause pancreatitis (Otsuki 2000). Second, in the choline-deficient, ethionine-supplemented model of pancreatitis in mice, CCK worsens the pancreatitis (Niedermaier 1985). Third, OLETF rats with a selective defect in the CCK-1 receptor develop less severe pancreatitis induced by taurocholate intraductal infusion, by a closed duodenal loop, or by intraperitoneal L-arginine administration (Tachibana 1997). Fourth, CCK-1 receptors antagonists were found to markedly reduce the severity of pancreatitis and improve survival in experimental models of acute pancreatitis (Niedermaier 1985, Niedermaier 1986, Niedermaier 1999, Satake 1999a, Satake 1999b). In men, a clinical double blind, not placebo-controlled, trial of intravenous infusion of the CCK-1-receptor antagonist, lorglumide, demonstrated a significant therapeutic benefit to patients with acute pancreatitis (Ochi 1999). A double-blind study comparing lorglumide with a trypsin inhibitor, nafamostat mesylate, also showed a

therapeutic effect in patients with acute pancreatitis (Satake 1999b). Double-blind placebo controlled studies are however still needed to clearly establish the role of CCK-1 receptor antagonists in the treatment of acute pancreatitis (Jensen 2002).

In chronic pancreatitis, levels of plasma CCK have been reported to be higher than those in control subjects (Schafmayer 1985, Gomez Cerezo 1991). The mechanism of this increase may rely upon the negative feedback regulation of pancreatic exocrine secretion: the lack in intraduodenal proteases due to the pancreatic exocrine insufficiency may increase CCK secretion. The increased CCK levels are supposed to stimulate pancreatic exocrine secretion, which induces high intraductal pressure, progression of the pancreatitis, and pain. It has indeed been demonstrated that elevated CCK levels were more common in patients with abdominal pain (Garces 1998). This pain in chronic pancreatitis is often severe and tends to persist. Diet supplementation with pancreatic enzymes has been proven in several studies to decrease pain (Toskes 2001). This treatment is based on the “feedback mechanism” of luminal enzymes on CCK and the LCRF (Owyang 1986a, Layer 1990, Jin 1994). Another CCK based treatment approach is to give these patients oral CCK antagonists. The clinical evaluation of oral administration of loxiglumide, a CCK-1 receptor antagonist, to patients with acute, painful attacks of chronic pancreatitis has recently been reported. A significant decrease in pain and serum pancreatic enzymes was observed as well as overall clinical improvement. These preliminary results indicate that this treatment may be useful in these patients (Shiratori 2002).

Some reports however were unable to confirm elevated CCK plasma levels in patients with chronic pancreatitis (Bozkurt 1988, Nustede 1991, Eddes 1999). The clinical impact of treatment with CCK antagonists remains therefore to be further evaluated in prospective and controlled trials. Caution should also be taken to differentiate the analgesic neural effects and the pancreatic secretory action of CCK antagonists. Loxiglumide was indeed reported to have analgesic effects in chronic pancreatitis, maybe mediated through opiate receptors (Rezvani 1987, McCleane 1998).

9.2 Gallbladder and bile ducts

9.2.a Gallbladder

CCK is the major hormonal stimulus of postprandial gallbladder contraction and sphincter of Oddi relaxation (Lin 1975, Behar 1980). The receptors found on gallbladder muscle bind CCK-58, CCK-33 and CCK-8 with similar potency (Eysselein 1983). These muscle cell receptors have been shown to be CCK-1 receptors by use of specific antagonists

(Schjoldager 1988). The physiological importance of correlations between CCK and gallbladder contraction have been supported by intravenous infusions of low doses of CCK-8 (Byrnes 1981, Shiratori 1986) and by specific antagonism of CCK-1 receptors in *in vivo* studies (Lotti 1987, Niederau 1989).

Although the concept that CCK acts as a hormone to cause gallbladder emptying is solidly established, the means by which this occurs have been the subject of considerable debate. Substantial support exists for the conventional view that CCK acts directly on smooth muscle cells to cause gallbladder contractions, but growing evidence suggests that a neural mechanism is more important physiologically. Hormonal cholecystokinin and sympathetic nerves can up- or down-regulate neurotransmission in the gallbladder, by altering the rate of acetylcholine release from vagal pre-ganglionic terminals. Peptides released from sensory axons can then act directly on gallbladder neurons to increase their excitability (Mawe 1991, 1998).

Bile flow does also seem to be stimulated by CCK through a ductular secretory mechanism, contributing to the overall process of delivery of bile to the duodenum (Douglas 1989a).

9.2.b Sphincter of Oddi

The sphincter of Oddi is even more responsive than the gallbladder to CCK given *in vivo* (Behar 1980). Relaxation of the sphincter of Oddi appears to be due to an indirect neural effect of CCK. Cholecystokinin acts on the sphincter of Oddi via pre-ganglionic cholinergic nerves to release vasoactive intestinal polypeptide and nitric oxide, and so lower tone. These events are co-ordinated with motility and secretory events in the upper gastrointestinal tract, delivering bile at appropriate times into the duodenum (Shaffer 2000).

9.2.c Clinical impact in biliary diseases

Pharmacologic intervention with CCK-8 is used primarily for the assessment of diseases of the gallbladder, common bile duct, and sphincter of Oddi dysfunction (Ziessman 2001). Infusion of CCK improves biliary imaging and can evoke pain in biliary motility disorders (Saremi 2002). Patients with symptoms consistent with biliary colic, who do not demonstrate calculi on routine sonography, present a diagnostic dilemma for clinicians. For those patients in whom other disease entities have been excluded and in whom the history and physical examination exemplify classic signs and symptoms of biliary disease, cholecystokinin cholescintigraphy or ultrasoundography with calculation of gallbladder ejection fraction may be a predictor of pathology as well as subsequent symptom relief after cholecystectomy (Barr 1997, Poynter 2002).

Patients with acalculous cholecystitis have impaired gallbladder contraction with infusion of CCK compared to controls or patients with pigment gallstones. A recent detailed study of the gallbladder muscle from patients with acalculous cholecystitis showed *in vitro* that the muscle had a defective response to CCK (Amaral 2001). The role of CCK in acalculous gallbladder disease remains controversial as well as the disease itself as a cause of chronic abdominal pain. A patient with obesity and gallstones was described with a 262 bp deletion in the 3rd exon in the CCK-1 receptor resulting in a receptor that did not bind CCK, but whether this contributes to the disease state remains unclear (Miller 1995b).

Cholecystectomy causes an exaggerated meal-stimulated CCK response. Since CCK is known to relax the lower oesophageal sphincter, these findings may help to explain the increased incidence of gastro-oesophageal reflux seen after cholecystectomy (McDonnell 2002).

Lack of CCK or CCK efficiency, e.g. due to antagonizing medications such as somatostatin analogs, has been shown to increase the risk of developing gallstone disease (Ewins 1992). On the contrary, CCK antagonists such as loxiglumide could be of some interest in the treatment of biliary pain, since CCK-1 receptors are involved (Beglinger 1989).

9.3 Stomach

9.3.a Gastric emptying

Gastric emptying after food ingestion is regulated by neural and hormonal factors. However, the relative contribution of each pathway is not yet clearly defined. CCK seems to play a crucial role in the lipid-induced regulation of gastric emptying and secretion (Kleibeuker 1988). It inhibits gastric emptying of nutrients by a complex action involving relaxation of the proximal stomach and contraction of the pylorus and proximal duodenum. The doses of exogenous CCK required to alter gastric motility are similar to those required for pancreatic and gallbladder effects (Schang 1981).

Experimental evidence for a role of CCK is best for gastric emptying of liquid meals that release CCK from the duodenum (Beglinger 1994). Administration of the CCK-1 receptor antagonist loxiglumide significantly accelerates gastric emptying of a liquid mixed and a glucose meal. The discrepant results observed with another CCK-1 receptor antagonist, MK329 (Liddle 1989), are difficult to explain considering the marked acceleration of gastric emptying rates shown in several animal studies. In the awake rat, lipid-induced inhibition of gastric emptying or gastric acid secretion is reduced by 70% by administration of a specific CCK-1 receptor antagonist (Holzer 1994). Although this cannot be tested in

humans, data from rats indicate that this action of CCK is mediated by CCK-1 type receptors on vagal afferent nerve terminals: functional ablation of the capsaicin-sensitive vagal afferent pathway appears to suppress around 70% of the responses to both CCK and lipid (Holzer 1994) and administration of the CCK-1 receptor antagonist in capsaicin-treated rats had no additional effect, suggesting that the CCK-mediated part of the response acts via the vagal capsaicin-sensitive pathway. Both extrinsic and intrinsic neurons express CCK-1 receptors and could thus be a target for CCK (Moriarty 1997, Sternini 1999). Consistent with the finding that they express CCK-1 receptors, extrinsic primary sensory neurons do seem to be directly sensitive to CCK as shown by electrophysiological recordings (Blackshaw 1990).

The first evidence showing that endogenous CCK is involved in the response of vagal afferents to nutrients was obtained by luminal application of casein acid hydrolysate on mesenteric nerve fibres. Fibres sensitive to casein acid hydrolysate were also sensitive to CCK, and responses to both were abolished by the CCK-1 receptor antagonist devazepide (Eastwood 1998). This is the first direct electrophysiological evidence to support the concept that CCK plays a role in the sensory transduction of luminal nutrient signals. The fact that casein hydrolysate does not increase plasma levels of CCK supports the local release of CCK. In distinct morphological studies, CCK-immunoreactive cells were localized ~10 to >100 µm from vagal afferent axons with no synapse like appositions, suggesting that CCK acts on nerves in a paracrine manner (Berthoud 1996). However, an alternative explanation is that CCK is released from intrinsic nerve terminals innervating the mucosa.

Taken together, current information favours the conclusion that CCK is involved in the physiologic regulation of gastric emptying and gastric motility in man. However, it seems that CCK mainly controls gastric emptying of the liquid but not the solid components. The pharmacologic effects of CCK-1 antagonists, which could be useful in the treatment of functional dyspepsia, gastroparesis and gastro-oesophageal reflux disease, need to be further investigated, in order to fully explore their potential as gastrokinetic drugs (Scarpignato 1993).

9.3.b Regulation of acid secretion

CCK peptides are weak stimulants of basal acid secretion in most species but inhibit stimulated acid secretion (Konturek 1993a). *In vitro* CCK-8 and gastrin caused equipotent stimulation of parietal cell secretion. Cholecystokinin has been shown to stimulate acid secretion directly and indirectly by binding to CCK-2 receptors on both parietal and enterochromaffin-like cells (Kopin 1992, Pisegna 1992). *In vivo* the inhibitory action of CCK could be mediated by selective release of an acid inhibitor. The candidate for

such an inhibition is somatostatin and CCK was shown to be more potent than gastrin to release somatostatin from D cells in culture (Soll 1985). This effect was shown to be mediated through CCK-1 receptors on D cells, which thereby exert a tonic inhibition on the parietal cells (Schmitz 2001a). In the CCK-1 receptor-deficient OLETF rats, the significantly higher basal acid outputs and stimulated acid outputs compared to those in control rats, further confirm the CCK-1 receptor mediated inhibition of acid secretion (Kanagawa 2002).

CCK can also be considered as an “enterogastrone” which means a hormonal substance released by fat in the intestine that causes physiological inhibition of gastric acid secretion. An important support of such a mechanism has been obtained by the use of specific CCK-1 receptor antagonists that markedly reduced acid inhibition caused by exogenous CCK in several species including humans (Lloyd 1992a, Lloyd 1992b, Konturek 1993b).

9.3.c Functional dyspepsia

The role of nutrient fat and the postprandial release of CCK in the development of dyspeptic symptoms were investigated in healthy volunteers and in patients with functional dyspepsia (Feinle 2001a, Feinle 2001b, Feinle 2001c). The studies showed that during distension of the stomach, lipids are a major trigger of dyspeptic symptoms such as nausea, bloating, pain, and fullness, and that they modulate upper gastrointestinal sensations and symptoms in a dose related fashion. CCK is a major mediator of the sensitisation of gastric perception by lipids in patients with functional dyspepsia as the CCK-1 receptor antagonist loxiglumide markedly diminishes this effect (Feinle 2002). Gastric relaxation in response to lipid was also abolished by a CCK-1 receptor antagonist (Borovicka 1996). In man, administration of a lipase inhibitor was shown to increase postprandial CCK levels but without affecting gastric compliance and sensitivity to distension and accommodation (Demarchi 2003).

These recent studies, although controversial, provide important insights into the mechanisms underlying gastrointestinal perception in response to fat and the role of CCK in patients with functional dyspepsia (Fried 2002). The pharmacologic effects of CCK-1 antagonists, which could be useful in the treatment of functional dyspepsia, gastroparesis and gastro-oesophageal reflux disease, need to be further investigated, in order to fully explore their potential as gastrokinetic drugs (Scarpignato 1993).

9.4 Motility effects on the oesophagus

The 2 types of CCK receptors have been described in the lower oesophageal sphincter (LOS): CCK-1 on

the inhibitory neurons and CCK-2 receptors on the smooth muscle (Rattan 1986). *In vitro*, CCK contracts the human lower oesophageal sphincter by stimulating muscular receptors. *In vivo*, CCK was shown to cause relaxation of the human LOS (Resin 1973), but plasma bioassay of CCK revealed that the amounts released in the circulation were insufficient to account for LOS relaxation caused by fatty meals (Brazier 1990). The CCK-1 receptor antagonist, loxiglumide, inhibited transient lower oesophageal sphincter relaxations (TLOSRS) and attenuated the fall in sphincter pressure following a meal, but had only modest effects on postprandial gastro-oesophageal reflux (Trudgill 2001).

TLOSRS are the main underlying mechanism of gastro-oesophageal reflux. Although CCK acts through CCK-1 receptors to increase the TLOSRS induced by gastric distension, the respective roles of endogenous CCK and fundic tone in triggering postprandial TLOSRS remain unknown. The effect of the CCK-1 receptor antagonist, loxiglumide, on postprandial LOS function and fundic tone in humans was recently studied and loxiglumide totally abolished the increase in TLOSRS, reduced LOS pressure fall, and strongly inhibited fundic relaxation. The authors concluded that endogenous CCK was involved in the postprandial control of both LOS function and fundic tone through activation of CCK-1 receptors (Zerbib 1998). Another study confirmed that endogenous CCK enhanced postprandial gastro-oesophageal reflux in humans by increasing the rate of TLOSRS and reduces postprandial LOS pressure. These actions seemed mediated by extra-sphincteric CCK-1 receptors that overrode a direct contractile effect of CCK on the LOS muscle (Clave 1998). CCK-1 receptor antagonists (loxiglumide in particular) might represent a pathophysiological approach to GORD therapy.

9.5 Motility effects on the small intestine

CCK analogues markedly accelerate the transit in the human small intestine (Bertaccini 1971, Levant 1974). CCK causes disruption of the fasting pattern of myoelectrical activity and produces contraction of the circular muscle and relaxation of the longitudinal muscle in some animal experimental models (Mukhopadhyay 1977, Stewart 1977, Weems 1985). The intestinal motility responses to CCK vary along the intestine according to the predominance of excitatory or inhibitory efferent innervation. Moreover, CCK-8 actions are mediated through both extrinsic and intrinsic afferent fibres (Giralt 1999).

The irritable bowel syndrome (IBS) is associated with motor abnormalities in the small intestine and colon. Neuropeptides may have an important role in initiating and regulating the intestinal motility. Motilin has been proposed to initiate the peristaltic

reflex in the small intestine and cholecystokinin the gastrocolic reflex. IBS patients have reduced motilin secretion after both water intake and a fat meal but, in contrast, fat meal elicited an exaggerated and prolonged CCK release. These data proposed that disturbed motilin and CCK release might partly be responsible for the intestinal dysmotility in IBS patients (Sjolund 1996). CCK antagonists could therefore be interesting pharmaceutical agents in IBS patients.

9.6 Motility effects on the colon

Colonic motor activity is controlled by the physico-chemical nature of the colonic contents, its degree of distension, neural mechanisms, and regulatory peptides, which act as hormones or neurotransmitters depending on their site of release (Sarna 1991b, a). Among peptides, CCK appears to play a major role. In *in vivo* human studies it has been shown that intravenous infusion of CCK or its analog caerulein increased colonic motor activity in the left or sigmoid colon (Dinoso 1973). CCK-8 could have an inhibitory effect in the proximal colon (Kellow 1987). Studies in rats and dogs, using CCK receptor antagonists, demonstrated that phasic motor responses to CCK-8 or caerulein were also different in the proximal and distal colon (Benouali-Pellissier 1994, Karaus 1995). All these studies only assessed variations of phasic motility recorded by electromyography or manometry using perfused catheters. In addition to phasic motor activity, the tonic component of the muscular activity, i.e., slow contraction or relaxation of the smooth muscle, is important to consider because it determines gut capacitance. Tonic activity can be recorded in the human colon by the electronic barostat (Steadman 1991). With the use of this technique in humans, dissociation between tonic and phasic activities and regional variations in tonic activity have been evidenced recently (Ford 1995).

The effects of CCK-8 on human colonic tonic and phasic activities after meal ingestion were assessed by using a tube assembly positioned in the cleansed transverse and left colon. No significant effects of intravenous administration of CCK-8 at supraphysiological levels could be shown in this study (O'Brien 1997). More recent data confirmed that intravenous administration of CCK-8 at physiological and supraphysiological levels modifies tonic and phasic activities of the unprepared colon in healthy subjects but did not support that CCK, acting as a hormone, was an important mediator of the colonic response to feeding (Coffin 1999). In irritable bowel syndrome, CCK antagonists, among multiple pharmaceutical agents, certainly deserve further clinical trials. Loxiglumide (and its dextroisomer, dexloxiglumide), for example, accelerated colonic transit, thereby increasing the number of bowel movements

in patients with chronic constipation and was also able to reduce visceral perception (Niederau 1992a, Meier 1993, Cann 1994, Scarpignato 1999, Sach 2002). Development and marketing of dexloxiglumide for the treatment of constipation-predominant IBS and phase III studies are currently ongoing in the US (Varga 2002).

9.7 CCK as a growth factor

9.7.a Pancreas

CCK can stimulate growth of normal and neoplastic tissues by interacting with CCK-1 or CCK-2 receptors. CCK administration has been shown to cause pancreatic growth in animals (Solomon 1978, Niederau 1987, Zucker 1989, Niederau 1994, Ohlsson 1999). Endogenous CCK-1 receptor activation assessed by the use of CCK-1 receptor antagonists results in stimulation of pancreatic weight in animals (rats, hamsters, mice) in some studies, but not others. Furthermore, mice that are CCK-deficient by target disruption of the CCK gene have similar pancreatic weights to wild-type controls fed a similar diet (Lacourse 1999), as do mice with a CCK-1 receptor knockout (Takiguchi 2002). These results suggest endogenous CCK or CCK-1 receptor activation is not essential to maintain pancreatic growth and perhaps compensation mechanisms exist to replace the loss of CCK.

9.7.b Pancreatic cancer

Numerous experimental studies in animals or *in vitro* studies of tumour cells have provided evidence that CCK-1 receptor activation may be important in both the induction and growth of cancers, especially of the pancreas (Guo 2000). CCK stimulates growth of some pancreatic cancers and inhibited the growth of others (Guo 2000). Prolonged elevation of CCK levels alone induces the formation of pre-neoplastic lesions in rat pancreas. CCK promotes the development of chemical carcinogen-induced pancreatic cancers in rats and hamsters. Studies *in vivo* with the receptor antagonist, lorglumide, show a decrease in the preneoplastic lesions produced by azaserine in rat pancreas (Douglas 1989b).

CCK has also trophic effects upon various cell lines derived from pancreatic exocrine glands or ducts. Several pancreatic cancer cell lines exhibited significant growth stimulation in response to low concentrations of CCK (Smith 1991). The rat pancreatic cancer cell line AR42J has been widely used to study proliferation induced by binding of CCK-1 and CCK-2 receptors (Seva 1990). In two studies, 90-100% of pancreatic adenocarcinomas expressed CCK-1 receptors (Weinberg 1997, Moonka 1999) and 100% of pancreatic adenocarcinoma cell lines (n=8) (Mandair

1998). However, even though CCK-1 receptors were present, CCK did not alter tumour cell function. In another study both CCK and CCK-1 receptor expression were not present in any pancreatic adenocarcinoma cell line studied, nor was CCK detected in the culture medium from any cell line evaluated, leading the authors to suggest CCK-1 receptor activation was unlikely to be involved in their growth (Monstein 2001).

The safety and efficacy of loxiglumide, a CCK-1 receptor antagonist, was evaluated in a randomised, placebo-controlled, double-blind study in patients with advanced pancreatic cancer (Militello 1997). Loxiglumide did not alter survival, even when patients were stratified by gender and stage. The effect of the CCK-1 receptor antagonist, MK-329, was also studied in patients with advanced pancreatic cancer. MK-329 had no effect on tumour progression, pain or nutritional status (Abbruzzese 1992). A more recent therapeutic trial with a highly selective, non-peptidic CCK-1 receptor antagonist (SR 27897B) has recently been published, unfortunately with negative results in terms of tumoral control (Eckel 2002). At present there is no study that unequivocally establishes that CCK-1 receptor activation is clinically important in human tumours.

9.7.c Other cancers

CCK-1 receptors have also been shown in other gastrointestinal cancers and are expressed in 38% of gastroenteropancreatic tumours, 19% of neuroblastomas, 30% of meningiomas, 0% of colon cancers, and 0-3% of other tumours (Clerc 1997, Reubi 1997). In general, CCK-1 receptor expression in human tumours is much less frequent than CCK-2 receptor expression. In one study using RT-PCR, 29% and 36% of gastric cancers were found to express CCK and CCK-1 receptor mRNA, respectively. Whether CCK or CCK-1 receptor activation effects growth of gastric cancer, is unknown (Okada 1996).

9.7.d CCK-2 receptors and gastrin

Gastrin has a growth-promoting effect on the oxyntic mucosa of the stomach but has been claimed also to affect other parts of the gastrointestinal tract, pancreas and liver. The effects of the CCK-2 receptor antagonists on various growth parameters in the stomach confirmed that these effects were mainly mediated by CCK-2 receptors (Bjorkqvist 2001). CCK-2 receptors are expressed in many tumours including 92% of medullary thyroid cancer (Blaker 2002), 57% of small-cell lung cancer (Staley 1990, Moody 2001), 65% of astrocytomas, 100% of stromal ovarian tumours and occasionally in gastrointestinal carcinoma (Clerc 1997), neuroendocrine tumours (Reubi 2001), breast, endometrial and ovarian carcinomas (Reubi 1997).

CCK-2 receptor-targeting peptides have been developed for staging and therapy of medullary thyroid cancer and other CCK-2 receptor-expressing malignancies (Behr 2002). There is considerable data on colon cancers containing CCK-2 receptors that mediate growth (Townsend 1989, Deprez 1995). CCK-2 receptor mRNA expression was demonstrated in 38% of human colorectal cancers and coexpression of receptor and ligand in 32% of tumours (Schmitz 2001b). Co-expression of receptor and ligand is a prerequisite for autocrine growth stimulation. In experimental protocols, the effect of hypergastrinaemia on tumour growth was reversed by use of antiserum directed against the CCK-2 receptor showing that hyper-gastrinaemia might promote proliferation of human colonic adenomas that express CCK-2 receptor isoforms (Watson 2002).

9.8 Various other effects

9.8.a Pepsinogen secretion

CCK also releases pepsinogen from isolated gastric glands and is more potent than gastrin for this effect. Excitatory inputs of CCK and gastrin to chief cells are not driven through acid-dependent mechanisms or capsaicin-sensitive afferent sensory nerves but probably directly on CCK-1 and CCK-2 cell receptors. Under *in vivo* conditions, the stimulant actions of CCK-like peptides on pepsinogen secretion are mediated, at least in part, by an increase in NO generation (Blandizzi 1999).

9.8.b Somatostatin release

Intravenous infusion of CCK-8 in rats caused a dose-dependent increase in portal venous somatostatin concentrations. These effects were reversed by the CCK-1 receptor devazepide but not by peri-vagal capsaicin (Lloyd 1992b). CCK also releases somatostatin in the following experimental models: isolated perfused canine pancreas, canine or rabbit D cells in primary culture or cultures of human antral mucosal cells (Buchan 1993, Arebi 2002).

9.8.c Pancreatic polypeptide release

CCK is also an effective releaser of pancreatic polypeptide in humans. Studies with CCK antagonists suggest that this effect is due to interaction with CCK-1 receptors and that CCK may play a physiological role in stimulation of pancreatic polypeptide release by meals (Lonovics 1980, Niederau 1992b).

10-Biological functions of CCK in the brain

10.1 General overview

Since its discovery in the brain, anatomical, physiological and pharmacological research on CCK and its possible involvement in neurological and psychiatric diseases has continued unabated. Vanderhaeghen et al rediscovered CCK in 1975 as one of the first gastrointestinal peptide found in the mammalian brain (Vanderhaeghen 1975). CCK is very abundant in the brain, more so than in the gut. To date, the only neuropeptide that is more abundant than CCK is neuropeptide Y. The major carboxyl-terminal amidated peptide in brain is CCK-8. Some CCK-4 has also been detected in rat brain. Larger forms like CCK-33, -39, and -58 have also been identified in the brains of some mammals (Qureshi 1993).

The anatomy of central CCKergic projections is very complex and has been the subject of intense investigation since its discovery. The ability to visualize CCK mRNA has provided much additional information about the location of CCK cell bodies. Many brain regions like the cortex and hippocampus contain both CCK-positive interneurons and a mixture of afferent and/or efferent cells and terminals. Certain regions like the striatum, nucleus accumbens and olfactory tubercle have abundant CCK terminals, but few CCK-positive cells. The expression of CCK is developmentally regulated. In the adult, several substances induce neuronal CCK mRNA expression via

activation of transcription factors binding to regulatory elements in the CCK promoter (Hansen 2001).

CCK is well established as a gastrointestinal hormone, but determination of the precise role of CCK in the brain has been more elusive. A large and rapidly expanding body of literature supports a neurotransmitter role for CCK. The neurobiochemical characteristics of CCK justify the claim that it can act as a classical neurotransmitter. It is an endogenous peptide, synthesised from procholecystokinin (proCCK). It is stored in synaptic vesicles in somas and nerve terminals from which it is released upon depolarisation in a calcium-dependent manner (Raiteri 1993). Much of the excitement about brain CCK is due to CCK's colocalization with dopamine and its ability to modulate dopaminergic neurotransmission. The presence of abundant CCK in the cortex and hippocampus colocalized with GABA and the CCK projections from prefrontal cortex to striatum have additional implications for a possible role in anxiety, attention deficit disorder, and in the negative symptoms and cognitive deficits of schizophrenia (Nair 1984) (Table 7).

10.2 CCK Receptor Subtypes, agonists and antagonists

The distribution of the CCK-1 and CCK-2 subtypes in brain is species-specific. In general, the CCK-2 receptor predominates in the brain, although significant populations of CCK-1 receptors are also present, and a number of physiological effects of CCK in the brain are mediated by CCK-2 receptors. Involvement of CCK subtypes in specific behaviours is frequently species-dependent (Hill 1987, Carlberg 1992).

Major questions remain about the site(s) of action of CCK agonists and antagonists after intraperitoneal,

Table 7

BIOLOGICAL FUNCTIONS OF CCK IN THE BRAIN

	CCK effect	Therapeutic target
Analgesia	Reduces opiate analgesia	CCK antagonists to increase morphine potency, manage drug addiction
Memory	Improves memory and attention tasks	
Satiety	Induces satiety -decreased gastric emptying -production of hyperglycaemia -antagonism of opiate system -central 5-HT receptors	? CCK analogs or antagonists
Anxiety	Induces panic attacks	? CCK antagonists as anxiolytic drugs

intravenous, or subcutaneous administration. It is unlikely that peripherally administered CCK-8 is entering the brain in significant quantities (Curry 1995): the penetration of some CCK-4 or CCK-5 is more likely. The possibility that small amounts of CCK-8 could be entering the brain in areas where the blood-brain barrier is leaky cannot be excluded. However, it is clear from many studies that peripherally administered CCK activates specific neuronal populations in areas where the blood-brain barrier is not leaky, such as the midbrain or the hippocampus (Skirboll 1981, Dahl 1987). Some of the antagonists, on the other hand, do penetrate the blood-brain barrier fairly well. The CCK-1 antagonist devazepide penetrates with an uptake index about one-half of that of diazepam (Pullen 1987). The CCK-2 antagonist CL-988, given subcutaneously or orally, blocks behavioural effects of centrally administered CCK (Hughes 1990).

10.3 Role of CCK in analgesia

The role of CCK in modulating opiate analgesia has been reviewed recently (Stanfa 1994). In rats, CCK acting on CCK-2 receptors reduces μ -opiate-mediated analgesia. In primates, CCK acting through CCK-1 receptors has similar actions in the spinal cord. CCK may be acting presynaptically to antagonize μ -opiate action by opposing μ -opiate-mediated suppression of the rise in internal calcium concentration produced by depolarisation of c-fiber terminals. This results in a decreased release of glutamate and neurokinins presynaptically. CCK may also be opposing opiate action by a similar mechanism postsynaptically.

CCKB antagonists increase the potency of spinal morphine and tend to reverse morphine tolerance (Dourish 1990). Changes in the levels of CCK or CCK release may participate in alterations of the effectiveness of morphine in inflammation or neuropathic pain models. CCK antagonists could thus be used to enhance morphine analgesia. The use of CCK-2 receptor antagonists can also be combined with enkephalin catabolism inhibitors in the management of pain and drug addiction (Roques 1996).

10.4 Role of CCK in memory

The corticostriatal pathway from the prefrontal cortex to the striatum is a major pathway implicated in attention deficit disorder and in the negative symptoms and cognitive deficits of schizophrenia. CCK is very abundant in the cortex and striatum and is probably a major transmitter in this pathway. In a recent study, unmedicated schizophrenic patients had significantly fewer dopamine receptors (Okubo 1997). Since CCK release is strongly influenced by dopamine receptor activation (Brog 1992), the CCK release in

these patients may be significantly decreased. Dopamine agonists (e.g., apomorphine) who increase CCK release are known to improve cognitive function in rat and primate models.

In a double-blind, cross-over study of normal men, caerulein improved selective attention tasks (Schreiber 1995). Feeding mice following a training session has been shown to increase memory (Flood 1989). This effect is mimicked by injection of CCK-8 and is blocked by cutting the vagus (Flood 1987) and by administration of a CCK-1 antagonist.

10.5 Role of CCK in satiety

CCK released from the duodenum after a meal is thought to be a physiological regulator of food intake (Silver 1991). This satiety-inducing effect of CCK was first reported in 1973, before the discovery of CCK in the brain (Gibbs 1973). During the next 30 years, significant progress in our overall understanding of the role of CCK in ingestive behaviour has been made. A physiologic role for endogenous CCK in the control of meal size has been demonstrated and sites and mechanisms of action for CCK in food intake have been investigated. Extensive studies in animals (Reidelberger 1991, Moran 2000) support the conclusion that CCK functions through gastrointestinal CCK-1 receptors as a satiety factor.

Peripheral CCK appears to act additively with other hormones like bombesin, somatostatin, calcitonin, and glucagon and with absorbed nutrients to produce termination of a meal, followed by the inter-meal interval. The precise mechanism by which CCK elicits satiety is not known, but decreased gastric emptying, production of hyperglycaemia, and antagonism of the opiate feeding system are just a few possibilities. This "peripheral" action of CCK is thought to be mediated by CCK-1 receptors located in the stomach, as sectioning of the gastric vagus prevents the satiety effect of peripherally administered CCK (Smith 1981). Studies of the effect of 5-HT antagonists on CCK-induced satiety suggest that also that central 5-HT receptors are involved in CCK-induced satiety (Stallone 1989). CCK release in the hypothalamus is increased after feeding in both rats and primates (Schick 1987), and central administration of CCK causes satiety (Schick 1994).

In several well controlled studies, CCK (at doses which produce the same circulating levels of CCK observed after a meal and which produce no adverse side effects) decreased food intake in experimental animals and in humans (Kissileff 1981, Crawley 1994). It is likely that CCK is acting both centrally and peripherally in a complex fashion with other factors like NPY, oestradiol, insulin, glucagon, serotonin, melanocortin and leptin in the regulation of feeding. Recently, it was demonstrated that exogenous CCK-8

rapidly mobilizes gastric leptin with a concomitant increase in plasma leptin concentrations (Bado 1998). Evidence was provided for a synergistic interaction between leptin and CCK leading to short-term reduction in food intake (Barrachina 1997). The food-reducing effect of leptin injected with CCK was specific and not associated with alterations in gastric emptying or locomotor behaviour. The leptin-CCK interaction was blocked by systemic capsaicin and L-364,718 but not by L-365,260, suggesting an involvement of CCK-1 receptors in the vagal afferents. It was further suggested that CCK may contribute to the long-term control of feeding and body weight when central leptin levels are elevated (Matson 1999).

Subsequent analyses using subtype selective agonists have suggested that the decrease in food con-

sumption resulting from exogenous cholecystokinin is mediated by the CCK-1 receptor (Crawley 1994). These investigations also suggested that stimulation of the CCK-2 receptor subtype plays no significant role in the inhibition of food intake resulting from exogenously administered peptide (Jensen 1994). Multiple studies have demonstrated that the CCK-1 receptor antagonist L-364,718 blocks the inhibition of food consumption resulting from exogenously administered CCK-8. Administration of L-364,718 alone to mice, rats, pigs, or monkeys increases food intake when assessed using a variety of feeding paradigms (Crawley 1994, Jensen 1994, Moran 2000). In addition, it was reported that L-364,718 enhanced hunger ratings in humans (Wolkowitz 1990). Despite the large body of evidence in support of endogenous CCK as a physiologic mediator of food intake, there

Table 8

SUMMARY OF CCK KNOCKOUT MOUSE STUDIES

	GENERAL	DIGESTIVE	BRAIN
CCK-1R DEFICIENT MICE			
CCK-1 knockout mice (Kopin 1999)	Viable Fertile Normal growth Normal behaviour	Gallstone disease	Conflicting results on NTS No CCK-induced inhibition of food intake Increased locomotor activity (Miyasaka 2002)
OLETF (Funakoshi 1995)	Obesity Diabetes	Pancreatic fibrosis Delayed gastric emptying (Ohta 2000)	
CCK-2R DEFICIENT MICE (Nagata 1996, Langhans 1997)			
	Viable Fertile Normal growth	Atrophy of gastric mucosa Decreased parietal cells (Friis-Hansen 1998) Achlorhydria Decreased ECL cells (Chen 2002) Hypergastrinemia	Reduction in memory and attention processes (Dauge 2001) Hyperalgesia and decreased sensitivity to opioids (Veraksits 2003) Enhanced locomotor activity Hyperanxiety (Miyasaka 2002)
CCK DEFICIENT MICE (Lacourse 1999)			
	Viable Normal growth	Increased amylase Lower brain and duodenal somatostatin	Not studied
GASTRIN DEFICIENT MICE (Koh 1997, Friis-Hansen 1998)			
	Viable Normal growth	Decreased parietal cells Achlorhydria Decreased colonic proliferation	Not studied

NTS : Nucleus tractus solitarius
CCK-1R : cholecystokinin-1 receptor
CCK-2R : cholecystokinin-2 receptor

are a considerable number of studies that have failed to detect a significant increase in feeding after L-364,718 administration (Khosla 1988, Gregory 1989, Dourish 1990, Smith 1992). The physiologic variables and/or subtle differences in the feeding paradigms, which explain the discrepant findings with this CCK-1 receptor antagonist are currently unknown. The understanding of endogenous cholecystokinin as a modulator of food intake was further complicated by results obtained with L-365,260, a CCK-2 receptor selective antagonist. The potency of L-365,260 in stimulating food intake, as well as in delaying the postprandial satiety sequence in partially satiated rats was reported to exceed that of L-364,718 (Dourish 1989). Although controversial (Corwin 1991, Reidelberger 1991, Moran 1992, Smith 1992, Weatherford 1992), this finding led to the hypothesis that under certain conditions, the CCK-2 receptor also plays a role in modulating food intake. As a possible mechanism, it was postulated that during stress or anxiety, CCK-2 receptor mediated pathways are activated and in turn may influence eating behaviour (Dourish 1992, Crawley 1994).

Targeted gene disruption has emerged as a powerful tool for assessing the role of a particular gene product in the maintenance of body weight and/or the development of obesity. The availability of mice lacking either CCK-1 or CCK-2/gastrin receptors provided an alternative approach to further address these issues (**Table 8**) (Inui 2000). Interest in the potential role of the CCK-1 receptors in regulating satiety and body weight was enhanced by a recent report that an inbred line of rats, Otsuka Long-Evans Tokushima Fatty (OLETF), lack CCK-1 receptors because of a spontaneous deletion of the corresponding gene. With aging, these animals develop obesity, diabetes, and pancreatic islet fibrosis with a strong male predominance of the phenotype (Miller 1995a, Moran 2000). OLETF rats express deficits in the control of the size of individual meals. Meal size in OLETF rats is doubled and although meal number is decreased, the decrease is not sufficient to prevent hyperphagia. Analyses of patterns of hypothalamic gene expression in OLETF rats suggest an important role for CCK in controlling NPY expression in a population of non-leptin regulated hypothalamic neurons. In the absence of this control, NPY is over-expressed, contributing to hyperphagia and obesity. Thus, the obesity in the OLETF rats may be the outcome of two regulatory disruptions, one depending upon a peripheral within meal satiety pathway and the other depending upon a central pathway critical to overall energy balance (Sheng 2002).

It is, however, not clear whether these abnormalities result only from the absence of the CCK-1 receptor, because OLETF rats appear to carry additional genetic defects (Kawano 1992, Funakoshi 1995, Takiguchi 1997, Moran 2000, Takiguchi 2002). It has however clearly been shown that CCK-1, and not

CCK-2 receptors, are involved in mediating the satiety effect of exogenous CCK (Kopin 1999). Administration i.p. of CCK-8 failed to decrease food intake in mice lacking CCK-1 receptors, whereas CCK-8 decreased food intake by up to 90% in both wild-type and CCK-2 receptor-deficient mice. However, CCK-1 receptor-deficient mice showed normal day- and night-food intake and body weight that is comparable to the corresponding age- and sex-matched wild-type controls (Kopin 1999). The similarity in weights persisted throughout the rapid growth phase and extended well into adulthood. CCK-2 receptor deficient mice also showed normal body weight but had a remarkable atrophy of the gastric mucosa demonstrating its importance in maintaining the normal cellular composition and function of the gastric mucosa (Nagata 1996, Langhans 1997). The loss of a functional CCK-2 receptor did not affect the expression of CCK-1 receptor mRNA in brain areas such as the cerebral cortex (Nagata 1996).

The therapeutic potential of CCK analogs cannot be estimated until further studies are performed that demonstrate the efficacy of analogs for decreasing body weight, and the safety of CCK when administered chronically. Because the brain-gut axis is so important in the normal control of food intake, a great deal of caution is needed, particularly with agents that will presumably be taken for prolonged periods. Even so, at present it seems likely that we will see the introduction of CCK analogs or CCK antagonists as a new class of appetite-modulating agents in the not-too-distant future (Beglinger 2002).

10.6 Role of CCK in anxiety

Much of the CCK (including the cortex and hippocampus) in the brain is co-localized with gamma amino butyric acid, GABA (Hendry 1984). CCK is known to stimulate GABA release (Rakovska 1995) whereas GABA inhibits CCK release (Raiteri 1996). The observation that the excitation of hippocampal neurons by CCK was antagonized by benzodiazepines revealed the possibility of involvement of CCK in anxiety (Bradwejn 1984). Some of the best CCK antagonists are modified benzodiazepine derivatives, a fact that supports this observation. Anecdotal evidence of panic-like symptoms following human administration of pentagastrin led to further investigations.

A number of well controlled human studies have shown that administration of CCK-4 or CCK-5 to panic attack patients or normal volunteers (de Montigny 1989) evoked panic-like attacks which were blocked by CCK-2 receptor antagonists (Bradwejn 1994) and the benzodiazepine anxiolytic lorazepam. Panic attack patients were more sensitive than normal volunteers to the effect of CCK-4, and preadministration of the CCK-2 receptor antagonist was shown to

inhibit the effect of CCK-4 (Bradwejn 1994).

This suggested that CCK antagonists might prove useful as anxiolytic drugs. However, initial human studies on endogenous anxiety did not confirm this. One clinical trial of the CCK-2 antagonist CI-988 found no beneficial effect, but the presence of a significant treatment-by-centre interaction and variable placebo response rate made the interpretation of the study results more difficult (Adams 1995). Another study, with L-354,260, did not find a significant effect either (Sramek 1994).

10.7 Summary

In the last few years, progress towards the elucidation of details of the biosynthesis, physiology and pharmacology of CCK has been made. The evidence that CCK plays a role in the neurochemical balance of the nervous system is well established. Elucidation of the precise nature of this role and determination of whether alterations in CCK function contribute to neurological and psychiatric disease are the next challenges. There is ample rationale from animal studies to begin and continue human testing of the new generation of CCK agonists and antagonists.

11-Causes of deficiency

11.1 Celiac disease

Defective CCK release has been found in patients with destruction of upper small intestinal mucosa due to celiac disease (Calam 1982). The reduced circulating concentration of CCK accounts for the diminished gallbladder contraction and pancreatic function observed in these patients (Braganza 1971, DiMagno 1972, DiMagno 1975a, Regan 1980, Fraquelli 1999). Appropriate CCK extraction methods and radioimmunoassays confirmed that this was related to a defective release rather than a lack of end-organ responsiveness to cholecystokinin (Low-Beer 1970, Braganza 1971, Low-Beer 1971, DiMagno 1972, Low-Beer 1974, DiMagno 1975a, Low-Beer 1975, Regan 1980, Masclee 1991, Fraquelli 1999). Gluten-free diet was also demonstrated to reverse the defective CCK release (Maton 1985).

The mechanism by which CCK release is impaired in coeliac disease is however still poorly understood. The main hypothesis is that the number of CCK cells and their hormonal content is decreased in the presence of mucosal atrophy (Calam 1982, Domschke 1989). Some immunocytochemistry reports showed

however increased number of CCK cells and increased CCK immunoreactive content in the celiac mucosa (Polak 1979, Sjolund 1979). Defective release or synthesis may therefore be related to other mechanisms: a lack of stimulation of the mucosal CCK cells due to impaired intra-duodenal hydrolysis of nutrients (Hopman 1995), a functional abnormality of the cell, an impaired relation with other intestinal cells, absence of a stimulating factor (e.g. luminal cholecystokinin releasing factor) or the presence of inhibitory factors associated with the inflammatory process taking place in the epithelium and the mucosa.

11.2 Bulimia, and various diseases associated with reduced CCK

Decreased plasma CCK concentrations have also been found in patients with bulimia nervosa, a condition characterized by abnormal feeding patterns (Geraciotti 1988). These patients have reduced meal-stimulated CCK release and this defect is resolved with tricyclic antidepressant-induced amelioration of the bulimic behaviour. A study showed that the decreased CCK release after a meal is associated with decreased gastric emptying. It was proposed that increased gastric capacity in these patients, perhaps resulting from repeated binge eating, gives rise to delayed gastric emptying and decreased postprandial CCK release which leads to an impaired satiety response, which may perpetuate the illness (Devlin 1997).

Patients with familial amyloid associated polyneuropathy have reduced intestinal endocrine cells and these changes may contribute to the development of intestinal motility dysfunction and maldigestion in the patients (el-Salhy 1994). Patients with a short bowel syndrome (Ling 2001) and diabetics (Bucceri 2002) were also described with low levels of CCK, but no role of this deficiency has been proven in the pathophysiology of these disorders. A V365I mutation in the CCK-1 receptor has been reported in obese, diabetic patients. Whether this mutation is contributing to either the diabetes mellitus or obesity, remains unclear (Marchal-Victorion 2002). Decreased brain concentrations of CCK have been found in schizophrenia and in Huntington's disease.

11.3 Autoimmune polyglandular syndrome type I

Cholecystokinin deficiency has been recently described in a patient with autoimmune polyglandular syndrome type I (Hogenauer 2001). The findings presented in this report suggested that a deficiency of cholecystokinin-producing enteroendocrine cells in the proximal small bowel caused severe malabsorp-

tion. The symptoms improved and worsened spontaneously. When the patient had severe malabsorption, postprandial plasma cholecystokinin concentrations were undetectable, and duodenal-biopsy specimens contained no enteroendocrine cells. When the malabsorption improved spontaneously, the serum cholecystokinin concentrations increased normally in response to food, and cholecystokinin-containing enteroendocrine cells were present in the duodenal mucosa.

Bile acid secretion, which is stimulated by cholecystokinin, was reduced when malabsorption was severe. Decreased bile acid secretion impairs the formation of micelles within the intestinal lumen and reduces the hydrolysis of dietary triglycerides by pancreatic lipase, thereby reducing the absorption of fat (Hofmann 1993, Pandol 1998). In this patient, the output of pancreatic enzymes was not as low as the output of bile acids, even though the secretion of both is regulated by cholecystokinin (Rehfeld 1989). These findings suggest that other mechanisms of pancreatic stimulation, such as vagal activity, may have caused some degree of pancreatic-enzyme output, even when stimulation by cholecystokinin was absent.

The cause of loss of enteroendocrine cells in this syndrome is unclear. Other gut hormones are affected (peptide YY and gastric inhibitory peptide). Tryptophan hydroxylase has been identified as a potential endogenous autoantigen. Cholecystokinin-producing cells also synthesize serotonin and therefore contain the enzyme tryptophan hydroxylase. The presence of tryptophan hydroxylase antibodies has been correlated with malabsorption and loss of serotonin-producing enterochromaffin cells in patients with autoimmune polyendocrine syndrome type I (Ekwall 1998). Since autoimmunity is believed to cause failure of the endocrine glands, it seems logical to surmise that autoimmunity mediated the destruction of small-bowel enteroendocrine cells in the patient. Although no serum autoantibodies were detected against normal enteroendocrine cells, this does not rule out the role of autoimmunity, because autoantibodies are not found in all patients with classic endocrine-gland failure due to autoimmune polyglandular syndrome type I (Betterle 1998).

The relationship between CCK deficiency and the clinical digestive symptoms in these patients has been questioned because CCK and CCK-1 receptor knockout mice do not have malabsorption and/or diarrhoea and because treatment with CCK-1 receptor antagonists never led to a reduction in pancreatic enzyme secretion severe enough to cause significant steatorrhea (Lembcke 1987, Creutzfeldt 2001, Schmitz 2001a).

11.4 CCK and CCK-receptors knockout mice

A deficiency of gut hormones or of their biological effects can be experimentally induced by several means: the suppression of hormone secretion by somatostatin, the use of specific receptor antagonists, and the generation of knockout mouse strains by targeted gene disruption (**Table 8**). All three approaches have been used to study the consequences of cholecystokinin deficiency in humans and animals. Subjects who received injections of the somatostatin analogue octreotide (Lembcke 1987, Ewins 1992) or oral treatment with the cholecystokinin antagonist lorglumide (Schmidt 1991) had gallbladder paresis but only slightly reduced secretion of pancreatic enzymes and mild steatorrhea (10 to 20 g of fat excreted). The use of specific receptor antagonists has been described earlier in this thesis. Mice with a deficiency of the CCK-1 receptor have normal food intake and weight gain (Kopin 1999). They are susceptible to gallstone formation but do not have diarrhoea or malabsorption (Lacourse 1999). CCK-2 receptor deficient mice also showed normal body weight but had a remarkable atrophy of the gastric mucosa demonstrating its importance in maintaining the normal cellular composition and function of the gastric mucosa (Nagata 1996, Langhans 1997).

CCK-deficient mice have been recently generated by gene targeting in mouse embryonic stem cells (Lacourse 1999). The targeting event disrupted the protein coding region by insertion of the bacterial lacZ reporter gene at the translational site. The deletion completely removed the NH₂ terminus of CCK, including the signal sequence and resulted in mice with no CCK measurable in brain and duodenum tissue extracts. CCK-deficient mice were viable and fertile and exhibited normal body weight. Pancreas weight and cellular morphology appeared normal, although pancreatic amylase content was elevated in CCK-deficient mice. CCK was shown not to be required for protein-induced pancreatic hypertrophy and increased proteolytic enzyme content, although CCK has been considered the primary mediator of dietary protein-induced changes in the pancreas. Altered somatostatin concentrations in brain and duodenum of CCK-deficient mice suggest that other regulatory pathways are modified to compensate for the CCK deficiency. Further studies analysis of CCK-deficient mice will be necessary to help elucidate CCK's contribution to digestive and neural physiology.



CELIAC DISEASE

Celiac disease, also known as celiac sprue, is characterized by intestinal malabsorption resulting from inflammatory injury to the mucosa of the small intestine after the ingestion of wheat gluten or related rye and barley proteins. A strict gluten-free diet is followed by clinical and histological improvement, and relapse occurs when dietary gluten is reintroduced (Trier 1991). This condition has been recognized from the early times, with Arateus the Cappadocian describing a chronic wasting illness associated with diarrhoea, general debility and “atrophy of the body” in the second century AD (Adams 1856). Samuel Gee described the condition as we know it in 1887. He noted the disease affected all ages and he thought that “to regulate food was the main part of the treatment”. It was however not until the 1940s, however, that the link to gluten ingestion was established. Dicke, a Dutch paediatrician, reported during World War II that affected children improved their nutritional status when cereal products were scarce and relapsed when these products became widely available again (Dicke 1950).

1-Epidemiology

The true prevalence of celiac sprue is difficult to ascertain, because many patients have atypical symptoms or none at all. Today, greater awareness of its presentations and the availability of accurate serologic tests have led to the realization that celiac sprue is far more prevalent in the general population than previously supposed, affecting 1 of every 120 to 300 persons (Mylotte 1973, Catassi 1996, Johnston 1998, Not 1998). A large, multicenter Italian study identified seven new cases of celiac sprue in children for each patient with established disease (Catassi 1996). The highest reported prevalence is in Western Europe and in places where Europeans emigrated, notably North America and Australia. Celiac sprue is also found in parts of northwest India, and it may be underdiagnosed in South America, North Africa, and Asia (Fasano 2001a). It is rare among people from a purely African-Caribbean, Chinese, or Japanese background. In most series there is a slight female preponderance.

2-Pathophysiology

The currently accepted theory of pathogenesis is that genetically predisposed people exhibit an inappropriate T-cell-mediated immune response against ingested gluten, and the resulting damage is caused by locally activated CD4+ T-lymphocytes (Schuppan 2000).

2.1 Abnormal permeability

The initial event in the pathogenesis of the celiac lesion is thought to be an abnormal permeability that allows the entry of gliadin peptides not entirely degraded by the luminal and brush border-bound peptidases. This increased permeability to macromolecules has been demonstrated some 30 years ago and was proposed as a screening test for the disease (Johnston 2000). A recent study demonstrated that improvement of intestinal permeability precedes morphologic recovery of the small intestine in celiac patients who begin a gluten-free diet (Cummins 2001).

This alteration of permeability was shown to be secondary to a loosening of the intestinal tight junctions and may represent, by allowing a quantitatively more meaningful entry of antigenic peptides, a sort of prerequisite for the further development of the immune events to come (Fasano 2000).

2.2 Genetic predisposition

The disease then develops in predisposed people: the importance of genetic factors is supported by the approximately 10 percent prevalence of the disease among first-degree relatives. A population-based twin study of celiac disease recently showed that concordance rates for celiac disease of 75% were found between monozygotic twins compared with 11% in all dizygotic twins (Greco 2002). About 95% of all celiac patients belong to the HLA-DR3 (or DR5/DR7 heterozygous) genotype and express the DQ2 α , β -heterodimer, encoded by DQA1*0501/DQB1*0201, whereas the remaining 5% are DR4 and show the DQ8 α , β -heterodimer, encoded by DQA1*0301/DQB1*0302 (Howell 1986, Howell 1988, Lundin 1994). DQ2 and DQ8 molecules are located on the surface of antigen-presenting cells and bind peptides to be presented to CD4+ T lymphocytes.

The low concordance rate for celiac disease in HLA-identical siblings is only 30%-50% and the risk for a first-degree relative of an affected person of developing celiac disease is in the order of 10-20%. Furthermore, 25% of non-affected Northern European population carries DQ2. These facts suggest that other non-HLA linked genetic influences may be necessary for the condition to develop. Several genome wide linkage studies and follow-up linkage studies of candidate regions (5q, 11q, 11p11) have been published during the last years. The only non-HLA locus to reproducibly show evidence for association with celiac disease between populations is the cytotoxic T lymphocyte-associated (CTLA-4)/CD28 gene region on 2q33 (King 2002). CD28 and CTLA-4 molecules are expressed by T lymphocytes and have a regulatory role on T cell function.

2.3 Interaction between the tTG modified gliadin peptide, HLA-DQ2 and T cells

Celiac patients exposed to gluten express high levels of serum antibodies to gliadin, endomysium, reticulins and tissue transglutaminase 2 (tTG2). The enzyme tissue transglutaminase is now known to be the major target of the autoimmune response in celiac sprue (Dieterich 1997). It represents a family of enzymes (Skovbjerg 2002), some of which are more specific for the papillary dermis (tTG3), as seen in dermatitis herpetiformis (Sardy 2002). Tissue transglutaminase

deamidates gliadin peptides, generating acidic, negatively charged residues of glutamic acid. Because negatively charged residues are preferred in positions 4, 6, and 7 of the antigen-binding groove of HLA-DQ2, deamidated gliadin elicits a stronger T-cell response (Molberg 2001). This was shown for both DQ2 molecules and DQ8 molecules. These sensitised T cells expressing the α / β T-cell receptor then activate other lymphocytes to generate cytokines, such as interferon- γ , interleukin-4, and tumour necrosis factor α (TNF- α), which damage the villi, resulting in enteritis. Induction of aberrant HLA class II cell-surface antigens on the enterocytes may permit these cells to present additional antigens to the sensitised lymphocytes.

The modification of gliadin by host tissue transglutaminase has thus a key role in enhancing the gliadin-specific T-cell response. A restricted number of gliadin (and glutenin) epitopes have been identified as toxic for the celiac mucosa. The influence of proline to protect against digestive proteolysis and to direct tTG2-mediated deamidation of glutamines contributes to this epitope clustering. A single tTG-modified peptide (residues 56-75 of α -gliadin, rich in proline) was recognized as the dominant epitope for T-lymphocytes in celiac patients. Gluten-specific T cells might help tTG2-specific B cells by linked recognition of tTG2-gluten-peptide complexes (Sollid 2002). This could explain how exposure to a foreign antigen controls the formation of autoantibodies. This may also represent an interesting target for future antigen-specific peptide therapy (Molberg 1998, Anderson 2000).

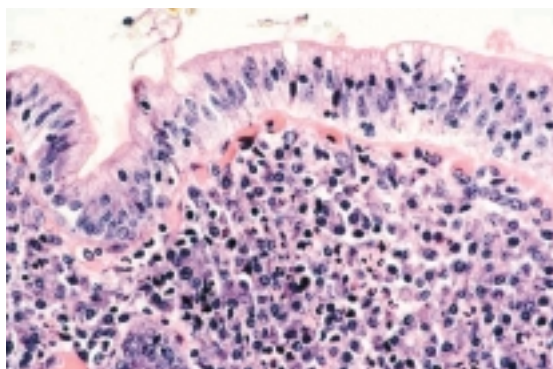
2.4 Events leading to mucosal lesions

2.4.a Morphological types of mucosal lesions

Celiac sprue has been defined more than 30 years ago in terms of the flat lesion, usually associated with a malabsorption syndrome that responds to a gluten-free diet (DiMagno 1969, DiMagno 1972). Recently, more refined descriptions of gluten-induced mucosal changes have been given that might be termed the spectrum of gluten sensitivity. At least five distinctive types of mucosal lesions can be recognised and have been called pre-infiltrative, infiltrative, hyperplastic, destructive and hypoplastic (flat) (Marsh 1992). The infiltrative lesion, seen in the latent phase, is characterized by morphologically normal mucosa and is not usually associated with gastrointestinal symptoms. This lesion occurs in 40% of patients with dermatitis herpetiformis and 10% of first-degree relatives of patients with celiac sprue. Initial changes seen include an increase in the number of intraepithelial lymphocytes (IELs), followed by infiltration of the lamina propria with plasma cells and lymphocytes (Figure 3). Even in histological changes of lesser degrees, some functional abnormalities have been

Figure 3

Intraepithelial and lamina propria infiltration of lymphocytes and inflammatory cells (Hematoxylin-Eosin x100).



reported, for example increased permeability (van Elburg 1993, Cummins 2001). The “normal” number of IELs in the duodenal mucosa has been a matter of debate for many years. A cut-off level of 25 IELs/100 epithelial cells has been recently proposed based on rigorous counting in individuals with normal permeability test and distal duodenal biopsy (Hayat 2002). Twelve or more IELs in the tips of several villi in architecturally normal biopsy specimens were even suggestive of celiac disease based on follow-up in patients suspected of having celiac disease (Goldstein 2001).

Additional features characterize the hyperplastic lesion, which is seen in 20% of celiac patients: elongation of crypts caused by a marked increase in undifferentiated crypt cells.

The destructive lesion is the classical celiac lesion and is synonymous with villous atrophy (Marsh 1993, Marsh 1995). It is found in 40% of patients with dermatitis herpetiformis and 10-20% of first-degree relatives of celiac patients. This lesion is characteristic of celiac disease but not diagnostic since it can be seen with severe giardiasis, infantile food sensitivities, graft versus host disease, chronic ischemia of the small intestine, tropical sprue, Ig deficiencies, and other immune deficiencies and allograft rejection (**Figure 4**).

The hypoplastic or flat lesion can be considered as the end-stage lesion in a very small group of patients who are unresponsive to gluten withdrawal and may develop malignant complications.

2.4.b Involvement of T cells

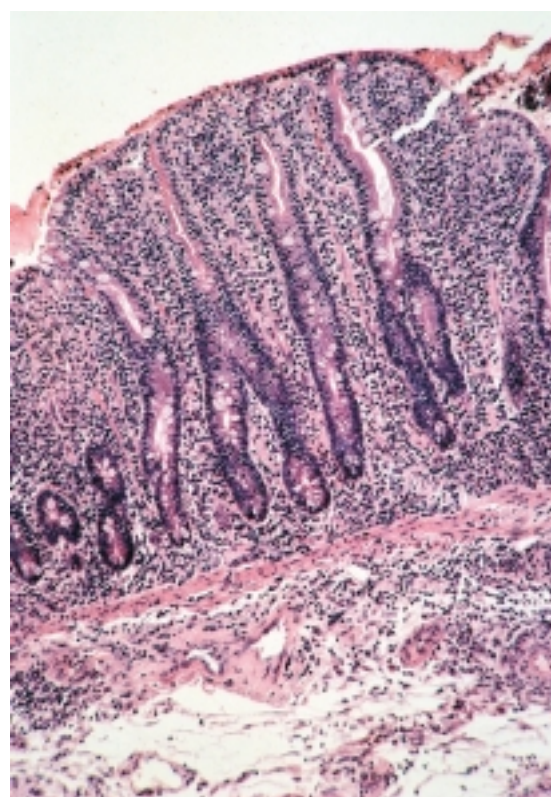
T cells in the gastrointestinal tract can be characterized anatomically and functionally into three compartments (James 1991). Luminal antigens first

encounter T cells residing between the intestinal epithelial cells: intraepithelial lymphocytes (IELs). The principal *in vitro* functions of IELs appear to be cytotoxicity and IFN- γ production. Beneath the epithelial layer is a loosely organized lamina propria compartment containing many types of inflammatory cells. Lamina propria lymphocytes (LPLs) are similar to peripheral blood lymphocytes phenotypically but express markers of prior T-cell activation such as CD45RO and the interleukin-2 receptor (Schieferdecker 1992). Functionally, LPLs are characterized by their proliferative and cytokine responses through the T-cell receptor (TCR) and enhanced reactivity through the CD2 activation pathway (Targan 1995). Finally, T cells populate the interfollicular zone of the Peyer's patches, where their dominant role is to aid in the differentiation of B cells towards Ig-A producing cells and the generation of T-suppressor cells responsible for the phenomenon of oral tolerance (Brandtzaeg 1989).

Much research has been carried out attempting to understand the intimate mechanisms by which T-cell activation, triggered by the presentation of toxic gliadin epitopes, leads to the mucosal changes. The

Figure 4

Typical atrophic mucosa with crypt hypertrophy and inflammatory infiltrate seen in celiac disease (Hematoxylin-Eosin x32).



early events have been nicely showed in *in vitro* studies on mucosal biopsy specimens from patients with celiac disease (Maiuri 2001b). Within one hour of gliadin challenge, HLA-DR expression is markedly increased on both villous enterocytes and adjacent lamina propria macrophages. These cells begin to migrate to more superficial mucosal layers: activated helper CD4+ T cells and macrophages migrate to the subepithelial compartment during the first 24 hours. HLA-DQ expression becomes upregulated on mononuclear cells and finally CD8+ T cells invade epithelial cells. The early events (within 1 hour), such as increased HLA-DR expression, occur before any involvement of T lymphocytes and are highly suggestive of a direct toxic effect of gliadin on epithelial cells and macrophages or a release of pre-stored cytokines. Suggestions have been made that cytokines, released at the mucosal level by the gliadin specific T cells, could be involved in the cascade of events leading to tissue damage (Monteleone 2001).

Celiac disease patients have indeed higher densities of IL-2, IL-4 and IFN- γ , TNF- α , TNF- β , IL-10, IL-1 β , and TGF- β positive cells in the lamina propria than controls (Przemioslo 1994, Kontakou 1995, Beckett 1996, Wassen 1996, Hall 2000, Salvati 2002). The expression of both Th1 (IFN- γ , IL-2) and Th2 (IL-4, IL-10) cytokines is seen in patients with active disease, implying activation of Th0 cells (Lahat 1999). These findings support the hypothesis that lamina propria T cells and macrophages, through their secretion of cytokines, play a central role in the pathogenesis of celiac disease. In experimental models, cytokines (i.e., TNF- α) have been shown to induce modifications reminiscent of the ones observed in celiac disease with mucosal destruction.

Additionally, higher densities of TNF- α and IFN- γ positive cells occur in patients with latent celiac disease and with high $\gamma\delta$ -TCR+ IELs (Westerholm-Ormio 2002). Up-regulated expression of migration inhibition factor (MIF) and TNF- α in IELs and epithelial cells of histological normal mucosa was also shown in patients with celiac disease on a gluten free diet (O'Keeffe 2001). The inflammatory cytokines found in patients with a disease limited to IELs infiltration strongly suggest that these inflammatory markers can be identified long before visible villous changes have occurred.

2.4.c Role of intraepithelial lymphocytes (IELs)

General description and function

Locally resident intraepithelial lymphocytes (IELs) are primarily T cells with potent cytolytic and immunoregulatory capacities, which they use to sustain epithelial integrity (Hayday 2001). IELs are found interspersed between the epithelial cells of the gastrointestinal tract. There are on average 10–20 IELs per 100 villous enterocytes in the small bowel in

humans (Ferguson 1971). Given the immense surface area of epithelia, resident IELs can comprise a substantial fraction of the body's T cells. They are the first immune cells to come in contact with antigens but their function in man is still not completely understood. Immunological studies on tissue sections have shown that IELs are phenotypically distinct from the lamina propria compartment (Hoang 1992b). Ninety to 95% of IELs are T cells (CD3+) and can be distinguished from peripheral blood lymphocytes by their high proportion of cytotoxic-suppressor CD8+ T cells (approximately 80%) (Lefrancois 1991). In human, 10–15% of IELs also express a T-cell antigen receptor (TCR) composed of γ - and δ -chains rather than the $\alpha\beta$ form of the TCR. These IELs are extra-thymically derived and the site of their maturation is within the intestinal epithelium. They are predominantly memory cells and express conventional T cell activation markers IL-2R (CD25) and class II HLA antigens.

The intimate anatomical relationship between IELs and intestinal epithelial cells suggests that the function of one is affected by the other in a manner that may have important pathological significance. Intestinal epithelial cells are able to activate IELs dependant on their expression of HLA molecules (Hoang 1997). Moreover it is possible that IELs could influence the function of epithelial cells since they do have potent down-regulatory properties. It is possible that in normal subjects the immunological cells of the lamina propria are kept in a “relative” state of immunological inactivity by such a signal emanating from the IELs. In certain circumstances such as celiac disease this negative tone imparted by the IELs to the underlying lamina propria may be lost, resulting in a chronic inflammatory response (Hoang 1997).

Type a IELs

More recently IELs were classified into just two cell types: “a” and “b” (Hayday 2001). “Type a” IELs include TCR cells that primarily recognize antigens presented by conventional MHC class I and II and are primed within the systemic circulation. “Type b” cells include TCR $\alpha\beta$ + CD8 $\alpha\alpha$ + IELs and TCR $\gamma\delta$ + IELs that respond to antigens not restricted by conventional MHC.

“Type a” IELs of the small intestine are primarily cytolytic, killing via granzymes or by engagement of *Fas* (Corazza 2000) and also secrete T helper type 1 (Th1) cytokines (Taguchi 1991). They are primed to antigen in gut-associated Peyer's Patches or in extrafollicular areas of the gut, after which they drain via mesenteric lymph nodes and lymphatics to the thoracic duct (Nanno 1994, Arstila 2000). After entering the blood, they home back to the lamina propria throughout the small and the large intestine. From the lamina propria they enter the epithelium with variable efficiency and may continue to exchange across the two compartments (Marsh 1975,

Ferguson 1977, Beagley 1998). Within the epithelium, particular “type a” clones will accumulate in response to repeated priming to an antigen that is expressed on enterocytes (Vezys 2000). Although the retention of such cells in the epithelium decreases with time, many are maintained in the apposite lamina propria, with the capacity to re-enter the epithelium (Masopust 2001). Compared to splenic memory cells, gut-associated T cells can be rapidly activated and may provide immediate cytolytic and cytokine-mediated responses.

Type b IELs

Like “type a” IELs, “type b” IELs are cytolytic and secrete cytokines and chemokines (Taguchi 1991, Barrett 1992, Lundqvist 1996, Corazza 2000). Because “type b” IELs do not recognize antigens presented on professional antigen presenting cells, it is conceivable that they are primed directly by epithelial cells *in situ*. Because the epithelium cannot provide the diversity of encounters with antigen that is provided in the lymphoid follicles, it is proposed that IELs are autoreactive and independent of specific foreign epitopes (Janeway 1988). In contrast to lymph node CD8+ $\alpha\beta$ T cells, which must be activated to become cytotoxic effectors, $\gamma\delta$ +IELs constitutively transcribe cytotoxic genes. $\gamma\delta$ +IELs also expressed natural-killer-inhibitory receptors, which might keep their effector functions under control, but allow them to be rapidly activated against a wide range of pathogens without transcriptional delays (Shires 2001). Interestingly, almost 25 years ago it was hypothesized that IELs might deal with antigen in an anti-inflammatory manner, whereas antigens that gained immediate and unregulated access to the systemic circulation would be pro-inflammatory (Ferguson 1977). There is recently some evidence that “type b” IELs might protect the epithelium integrity, towards gut infection for example (Roberts 1996). Some results have also suggested that $\gamma\delta$ +IELs contribute to oral tolerance (Fujihashi 1990, Ke 1997). $\gamma\delta$ +IELs may modulate local immune responses and participate in intestinal lipid metabolism, cholesterol homeostasis, and physiology (Fahrer 2001).

IELs and celiac disease

A significant clue to defining the reactivities and functions of type b IELs must be their dramatic expansion (particularly of $\gamma\delta$ +IELs) in celiac disease, a wheat protein allergy that provokes CD4+ $\alpha\beta$ T cell-mediated erosion of the small intestinal villi (Ferguson 1971, Ferguson 1977). Clearly, celiac disease is characterized by an intense infiltration of activated T cells, but the prospect that “type b” IELs respond to these alone does not obviously explain why “type b” IEL numbers are not amplified in other gut immunopathologies. The gliadin peptides that pro-

voke the allergy can be directly enteropathic, and it is conceivable that “type b” IELs respond to a pathogenic change in the enterocytes. By whatever means, the end-result of “type b” IEL activation may again be to suppress tissue infiltration by systemic T cells, as peak “type b” cell representation seems to correlate inversely with disease symptoms (Hayday 1994).

Functions of IELs

Although their precise role *in vivo* remains enigmatic, it has been shown *in vitro* that, under certain conditions, human IELs exert various forms of cytotoxicity, are capable of proliferation, can suppress IgA synthesis and perform some regulatory functions (Cerf-Bensussan 1985, Ebert 1989, 1990, Hoang 1991, Hoang 1992a, Ebert 1993, Sachdev 1993, Sperber 1995). It is believed that they may communicate with the adjacent epithelial cells and lamina propria cells (Watanabe 1995, Hoang 1997). It has been shown that IELs produce IFN- γ , IL-2, IL-3, IL-5, IL-6, IL-10 but not IL-4 (Taguchi 1991, Barrett 1992, Forsberg 2002). The production of some cytokines by IELs is markedly increased after polyclonal activation or stimulation through the CD2 molecules (Ebert 1990). Freshly isolated IELs express high levels of IL-1b, IL-8, IL-2R and IFN- γ mRNA and significantly less IL-1 α , IL-6, IL-7 and TNF- β (Deusch 1991, Lundqvist 1995). More recently, experimental data clearly indicate that IELs migrate into the epithelium with the assignment of killing enterocytes, by inducing apoptosis (Ciccocioppo 2000, Jabri 2000).

Gene-expression profile of IELs

A more detailed description of IELs is necessary if one is to understand their roles in down-regulating infection, transformation and systemic infiltration of the local environment, as well as their involvement in human disease. Two recent studies that investigated the gene-expression profile of IELs provide new insight into the function of these cells. IELs were revealed as cytolytic Th-1-skewed immunoregulatory cells, in keeping with earlier studies (Taguchi 1991, Barrett 1992, Lundqvist 1996, Corazza 2000). Specific results, including the expression of cytolytic mediators (such as, granzymes and *Fas* ligand), chemokines, chemokine receptors and adhesion molecules were likewise predictable. A second observation was the selectivity of effector molecule expression. Although the expression of chemokines that recruit T cells and APCs was very high, the expression of chemokines that recruit neutrophils was negligible (Shires 2001). It is tempting to relate this to the anti-inflammatory effects of “type b” IELs. Other than the constitutive expression of lymphotoxin- β , TGF- β and the gut-associated cytokine IL-17B, there was negligible expression of conventional cytokines such as IL-2 and IFN- γ . The under-representation of IFN- γ , which is

produced by IELs (Taguchi 1991, Barrett 1992, Boismenu 1994), suggests that IELs require additional activation *in vivo* to manifest full functional potential. For this reason, we have termed the constitutive state of the IEL compartment as "activated yet resting" (Shires 2001). An "activated yet resting" state agrees with the scarcity of mitotic IELs *in situ* (Ferguson 1977), with the report that most extracted IELs are in G0 or G1 cycle phase (Yamamoto 1994) and with the constitutive activation of mitogen-activating protein (MAP) kinase in non-proliferating IELs but not systemic cells (Sydora 1993).

Provocatively, the disjunction between the high expression of cytolytic effectors and the rare expression of cytokines emphasizes the independent regulation of the two pathways. Other studies have likewise shown that IELs respond to CD3-TCR ligation with strong cytolytic, but weak proliferative, responses (Sydora 1993, Yamamoto 1994). For these reasons, Hayday et al proposed that IELs in the small intestine respond to activation in two steps. The first step involves the release of cytolytic mediators and immunoregulatory chemokines, whereas the second step will involve the up-regulation and release of cytokines, primarily Th-1 cytokines.

2.4.d Epithelial cell apoptosis

Celiac lesion may, in fact, be patchy along the duodenum and features of T-cell activation are usually detected, even in areas without morphologic abnormalities. A strict correlation between villous flattening, enterocyte expression of *Fas* and apoptosis is seen in the celiac duodenum. The element that differentiates patchy lesions with normal areas is the specific lack of *Fas* expression in the normal areas. *Fas* expressed in enterocytes can deliver a functional signal crucial in driving epithelial apoptosis. Blockage of *Fas* engagement in organ culture of celiac duodenal explants does control gliadin-induced epithelial apoptosis (Di Sabatino 2001).

The role of IELs in inducing epithelial cells apoptosis has been recently shown in patients with untreated celiac disease. A specific increase of IELs expressing CD24 has been shown in the duodenal mucosa of these patients and IL-15 was identified as a likely candidate for inducing CD94 expression (Jabri 2000). IL-15 is elaborated by monocytes or epithelial cells and seems to be involved in both the proliferating and atrophic phases of celiac disease. In organ culture models, IL-15 could induce proliferation in crypts and *Fas* expression in enterocytes and was also able to mediate intraepithelial infiltration of T lymphocytes (Maiuri 2000, Maiuri 2001a, Maiuri 2001b). Such lymphocytes express *Fas*-ligand, thus triggering apoptosis of *Fas*-positive enterocytes (Ciccocioppo 2000). The control of gliadin-induced epithelial apoptosis by neutralizing anti-*Fas* monoclonal anti-

bodies fails to control proliferation in crypt enterocytes, suggesting that proliferation may actually precede villous atrophy (Maiuri 2001a).

3-Clinical Manifestations

3.1 Celiac Sprue in Children

Classically, infants with celiac sprue present between the ages of 4 and 24 months with impaired growth, diarrhoea, and abdominal distension (Catassi 1997). Vomiting is common in young infants, as are pallor and oedema. The onset of symptoms is gradual and follows the introduction of cereals into the diet. The velocity of weight gain slowly decreases before weight loss ensues. Patients with severe, untreated celiac sprue may present with short stature, pubertal delay, iron and folate deficiency with anaemia, and rickets. Atypical celiac sprue is usually seen in older children or adolescents, who often have no overt features of malabsorption. In addition to recurrent abdominal pain, hypertransaminasemia, recurrent aphthous stomatitis, arthralgia, and defects in dental enamel, children may have behavioural disturbances such as depression, may be irritable, and may perform poorly in school.

3.2 Celiac Sprue in Adults

The diagnosis of celiac sprue is increasingly being made in adults. About 20 percent of cases occur in patients who are older than 60 years of age (Hankey 1994). Many adults present with episodic or nocturnal diarrhoea, flatulence, and weight loss. Enteropathy often results in symptomatic lactose intolerance. Abdominal discomfort and bloating are common and often lead to a mistaken diagnosis of irritable bowel syndrome. Malaise and lassitude are also common even when anaemia is absent. Recurrent aphthous stomatitis may be the sole symptom at presentation. Approximately 50 percent of adult patients do not have clinically significant diarrhoea.

Iron-deficiency anaemia is now the most common clinical presentation in adults with celiac sprue. Other laboratory abnormalities include macrocytic anaemia due to folate (or, rarely, vitamin B12) deficiency, coagulopathy resulting from vitamin K deficiency, or vitamin D deficiency leading to hypocalcaemia and an elevated alkaline phosphatase level (Cellier 2000b). Other increasingly recognized extraintestinal manifestations include osteomalacia, osteoporosis and bone fractures (Vasquez 2000), infertility (Collin 1996), psychiatric syndromes (De

Santis 1997), and various neurological conditions, including peripheral neuropathy, ataxia, and seizures (Hadjivassiliou 1996). The neurological conditions are immune mediated and not caused by nutritional deficiencies (Hadjivassiliou 2002).

3.3 Associated Conditions

Autoimmune diseases occur more commonly in patients with celiac sprue, especially type 1 diabetes mellitus (Cronin 1997, Sjoberg 1998) and autoimmune thyroiditis (Counsell 1994). The prevalence of celiac sprue in patients with type 1 diabetes is approximately 3 to 8 percent (Cronin 1997, Sjoberg 1998). Unexpected episodes of hypoglycaemia or diarrhoea should alert clinicians to the possibility of coexisting celiac sprue in patients with type 1 diabetes.

The duration of gluten exposure is associated with the prevalence of associated autoimmune diseases (primary biliary cirrhosis, primary sclerosing cholangitis, thyroid disease...) which is additional rationale for early diagnosis and treatment of celiac sprue (Ventura 1999). Many conditions occur in association with celiac sprue (Collin 1994). Dermatitis herpetiformis, epilepsy with cerebral calcifications, rheumatoid arthritis, IgA mesangial nephropathy and Down's syndrome are conditions in which an active search for celiac disease has been proposed.

4-Diagnosis

4.1 Serologic Tests

The serologic tests are used to evaluate patients with suspected disease, monitor adherence and response to a gluten-free diet, and screen patients with atypical, extraintestinal manifestations (Fasano 2001b). IgA antiendomysial antibodies are usually detected by indirect immunofluorescence with the use of sections of human umbilical cord or, less commonly, monkey oesophageal smooth muscle (Volta 1995). The reported sensitivity and specificity of antiendomysial antibodies are 85-98 percent and 97-100 percent, respectively (Ferreira 1992, Grodzinsky 1994, Volta 1995, Fasano 2001b).

Tissue transglutaminase is the autoantigen recognized by antiendomysial antibody (Dieterich 1997). New tissue transglutaminase assays have been developed and seem to be as sensitive and as specific as the antiendomysial antibody assay (Dieterich 1998, Sulkanen 1998, Baldas 2000). Although false positive

results are rare, false negative antiendomysial and tissue transglutaminase antibody results can occur in mild enteropathy, in children under two years of age, and especially in patients with IgA deficiency. Tests for IgA and IgG antigliadin antibodies have moderate sensitivity but are far less specific than tests for IgA antiendomysial antibodies (Uibo 1993, Rostami 1999). A test for IgG antigliadin antibody is useful in the 2 to 10 percent of patients with celiac sprue who have coexisting IgA deficiency.

Levels of IgA antigliadin, IgA antiendomysial, and IgA tissue transglutaminase antibody all become undetectable in patients who are on a strict gluten-free diet. Tests for IgA antigliadin antibody are useful to monitor dietary compliance, since levels of this antibody are the easiest to quantify (Fotoulaki 1999). Levels of IgA antigliadin antibody gradually become undetectable within three to six months after gluten is withdrawn from the diet.

4.2 Haematological and Biochemical Tests

Combined iron and folate deficiency is a characteristic consequence of enteropathy of the proximal portion of the small bowel but is less frequent than iron-deficiency anaemia. Deficiencies of iron, folate, calcium, and vitamin D may be found in patients with untreated celiac sprue. Features of hyposplenism (Howell-Jolly bodies and thrombocytosis) are frequently seen in older patients with untreated sprue (O'Grady 1984). A prolonged prothrombin time due to vitamin K deficiency is uncommon but should be corrected before a small-bowel biopsy is performed. Celiac sprue should also be considered in patients with persistent hypertransaminasemia (Novacek 1999).

Only patients with extensive and severe enteropathy will have evidence of steatorrhea on a microscopic evaluation of the stool or a three-day measurement of faecal fat (Holmes 1988). Similarly, although the results of an oral D-xylose-absorption test may be abnormal in patients with untreated celiac sprue, the test will not provide a specific diagnosis and is normal in many patients with mild-to-moderate enteropathy (Kelly 1990).

4.3 Biopsy of the Small Intestine

Upper gastrointestinal endoscopy has become a common procedure for celiac patients mainly to provide distal duodenal specimens for histology. Various markers of sprue have been described, including a reduction in the number or an absence of duodenal folds, the presence of scalloped folds and the mosaic appearance of the mucosa (Brocchi 1988, Mainguet

1989, Corazza 1990, Ravelli 2001). Videoendoscopy and dye staining of the mucosa have improved the endoscopic diagnosis of villous atrophy (Niveloni 1998). Endoscopic markers were however found disappointing or insensitive in the adult population with mild disease (Shah 2000, Dickey 2001, Oxentenko 2002). Histologic examination of a biopsy specimen of the small intestine remains the diagnostic gold standard for celiac sprue. Specimens should be obtained from the distal duodenum (second or third part) to avoid the architectural distortion produced by Brunner's glands or peptic duodenitis.

The classic lesion in patients with untreated celiac sprue is characterized histologically by striking mucosal architectural changes, with absent villi and hyperplastic crypts (Rubin 1968). There are increased numbers of intraepithelial lymphocytes and of plasma cells and lymphocytes in the lamina propria. It is noteworthy that the increase in IELs is the earliest detectable alteration in the mucosa, prior to the increase in lamina propria lymphocytes or architectural changes (see above). The finding of a raised IEL count with normal villous architecture is now considered of sufficient clinical importance to be highlighted in routine duodenal biopsy reports (Mahadeva 2002).

Increased IEL counts could also be used as a screening tool, complementary to serological testing (Leon 2002). Celiac disease is the only entity in which $\gamma\delta$ -IEL have been described as systematically, permanently, and markedly raised, compared to other conditions like food allergy. The combined study of total, $\gamma\delta$ +, and NK-like IELs, that could be termed "IEL lymphogram", is claimed to allow for nearly 94% specificity and sensitivity in the diagnosis of celiac disease after clinical suspicion (Camarero 2000). This technique shows its real value in latent and potential presentations of the disease, and offers important data for the differential diagnosis from other enteropathies. The severity and extent of the histologic abnormalities in celiac sprue vary indeed widely. Patients who have mild, focal abnormalities confined to the proximal small intestine are likely to have fewer symptoms and less malabsorption than patients with severe, extensive enteropathy.

4.4 Repeated Biopsy and Gluten Challenge

Although small-bowel biopsies are recommended to establish the diagnosis, the availability of accurate serologic tests has reduced the need for a second biopsy session. A second biopsy session can now be reserved for selected patients who have an unsatisfactory or equivocal clinical response to a strict gluten-free diet. In current practice, gluten challenge is reserved for the few patients in whom the diagnosis remains in doubt after a period of gluten restriction

(e.g., patients who began to follow a gluten-free diet empirically without documentation of the characteristic intestinal lesion or of the presence of antiendomysial or tissue transglutaminase antibodies).

Gluten challenge should also be considered if a diagnosis of celiac sprue was made during childhood on the basis of a small-intestine biopsy without a positive test for antiendomysial antibodies, since a number of transient childhood enteropathies can mimic celiac sprue histologically (Trier 1998).

4.5 Genetic testing

More than 95% of celiac patients share the major histocompatibility complex II class human leukocyte antigen (HLA) DQ2 or DQ8 haplotype; patients negative for both types are unlikely to suffer from celiac disease (Howell 1986, Howell 1988, Lundin 1994). But it should be recognized that 25% of "normal" Northern European population carries DQ2. Genetic testing has therefore no role in the diagnosis of celiac disease but due to its high negative predictive value HLA-DQ2 and -DQ8 determination is useful in exclusion, probably lifelong, of celiac disease in individuals with an equivocal small bowel histological finding (Sacchetti 2001, Kaukinen 2002). The low specificity of this test must, however, be borne in mind.

5-Treatment

Because a gluten-free diet represents a lifetime commitment, is more expensive than a normal diet, and may limit patients socially, especially children and teenagers, it should never be recommended unless the diagnosis of celiac sprue is firmly established. There is no role for an empirical therapeutic trial of gluten withdrawal because a patient's response is often equivocal and because the abnormal findings on both the serologic tests and small-bowel biopsy may revert to normal, making subsequent definitive diagnosis difficult (Trier 1997).

Products that contain wheat gluten or are produced from barley or rye must be avoided. In reality, complete elimination of gluten is very difficult to achieve and maintain. Gluten is present in many processed foods because wheat flour is widely used as a thickener in many commercial products and convenience foods. The institution of an effective gluten-free diet requires extensive, repeated counselling and instruction of the patient by the physician and dietician. Although the long-term safety of oats in adults or children with celiac sprue or dermatitis herpetiformis

is unknown, consumption of moderate amounts of oats (50 to 70 g per day) for 6 months to 5 years is non-toxic (Janatuinen 1995, Hardman 1997, Hoffenberg 2000, Janatuinen 2002). However, oat products may be contaminated with small amounts of wheat. Consequently, oats should be avoided in all patients with newly diagnosed celiac sprue until remission is achieved through the use of a gluten-free diet. Then, up to 50 gr of oats from a reliable source can be eaten every day and continued if the patient has no ill effects.

Dairy products should be avoided initially because patients with untreated celiac sprue often have secondary lactase deficiency. After three to six months of treatment, dairy products can be reintroduced if the patient has no ill effects.

Approximately 70 percent of patients have symptomatic improvement within two weeks after starting a gluten-free diet (Pink 1967). The speed and eventual degree of histologic improvement are unpredictable but invariably lag behind the clinical response and may not be evident on repeated biopsy for two to three months (Grefte 1988). Although a return to normal histologic findings is common in children, most (79%) adults have only a partial resolution on biopsy and present with partial or total mucosal atrophy at medium or long-term follow-up (Lee 2003). If a patient has no response to the diet, the most common cause is incomplete adherence. Persistent symptoms may be caused by coexisting disorders such as irritable bowel syndrome, lactose intolerance, microscopic colitis, or pancreatic insufficiency. In a large Finnish study the five-year survival rate among patients who strictly adhered to a gluten-free diet was similar to that of the general population (Collin 1994). Growth and development in infants and children proceed normally with continued avoidance of gluten.

In addition to a gluten-free diet, all patients with newly diagnosed celiac sprue who have clinically evident malabsorption should initially receive appropriate supplements to correct any iron or folate deficiency. Patients with steatorrhea, hypocalcaemia, or osteopenic bone disease should receive oral calcium and vitamin D supplementation. Patients with hyposplenism should receive prophylactic antibiotics before undergoing invasive manipulations and may benefit from pneumococcus vaccination. Occasionally, intravenous corticosteroid therapy is required for critically ill patients with acute celiac crisis, manifested by severe diarrhoea, dehydration, weight loss, acidosis, hypocalcaemia, and hypoproteinaemia (Lloyd-Still 1972).

6-Complications: refractory sprue and lymphoma

Refractory sprue is a diagnosis of exclusion, defined as symptomatic severe enteritis that does not respond to at least six months of a strict gluten-free diet and is not accounted for by other causes of enteropathy or by overt intestinal lymphoma (Ryan 2000). Patients with refractory sprue are at high risk for complications such as enteropathy-associated T-cell lymphoma, ulcerative jejunoileitis, and collagenous sprue. Almost 75 percent of such patients have an aberrant clonal intraepithelial T-cell population, a condition referred to as cryptic intestinal T-cell lymphoma (Cellier 2000a). These cells have destructive properties related to their cytotoxic phenotype, which lead to mucosal ulceration, lymph-node cavitation, and frequently, cellular and clinical progression to lymphoma (de Bruin 1997).

Patients with refractory sprue may require treatment with corticosteroids and other immunosuppressants, including azathioprine or cyclosporine, total parenteral nutrition, recombinant IL-10 or infliximab (Rolny 1999, Vaidya 1999, Mulder 2001, Gillett 2002). In one study strict adherence to a gluten-free diet reduced the risk of all disease-associated cancers including enteropathy-associated T-cell lymphoma (Holmes 1989). Thus, it seems prudent to recommend lifelong strict adherence to a gluten-free diet in all patients with celiac sprue.

The prevalence of carcinoma of the gastrointestinal tract in celiac sprue is more common than in controls, particularly small intestinal carcinoma, oesophageal and pharyngeal squamous carcinoma (Holmes 1989).

7-CCK deficiency and pancreatico-biliary dysfunction

Although ingestion of gluten and consequent small intestinal inflammation or villous atrophy is one possible cause of chronic diarrhea in celiac patients, there are other conditions epidemiologically linked to celiac sprue that can cause diarrhea. These include microscopic colitis (lymphocytic or collagenous), exocrine pancreatic insufficiency, diabetes mellitus, autoimmune thyroid disorders, immunoglobulin deficiency and lactose intolerance (Fine 1997).

There is considerable evidence that pancreatic dysfunction may be associated with celiac sprue (Regan 1980, Carroccio 1994, Gray 1997). Forty years ago, Dreiling reported that the pancreatic secretory response to secretin was muted in some patients with sprue (Dreiling 1957). Subsequent comprehensive analyses of exocrine pancreatic function in celiac sprue by DiMagno's group showed low secretion of pancreatic enzymes in about one third of patients with untreated sprue after stimulation with test meals and intraduodenal amino acids (DiMagno 1972). The response to exogenous CCK was abnormal in only some of these patients, suggesting that the major defect in untreated celiac sprue may be impaired release of endogenous CCK. In addition, the pancreatic insufficiency in celiac patients is probably also a consequence of the associated protein malnutrition that interferes with the high level of protein synthesis required for regeneration and secretion of the pancreatic digestive enzymes (Tandon 1969).

The pancreatic insufficiency seems to play an important role in determining the degree of growth recovery in children with celiac disease (Carroccio 1997). Fortunately, pancreatic function returns to normal when the intestinal capacity for surface digestion and transport is restored in celiac disease (Regan 1980). Reduced release of pancreatic enzymes after diagnosis of celiac disease can however still be

observed several months after gluten free diet (Carroccio 1991). The determination and monitoring of stool chymotrypsin has been suggested to identify patients with celiac disease who could benefit from pancreatic enzymes (Carroccio 1995).

Gallbladder inertia and sluggish enterohepatic circulation of bile salts further contribute to steatorrhea in celiac patients. Gallbladder inertia has been demonstrated in celiac patients by ultrasonography, oral cholecystography, cholescintigraphy and bilirubin output in response to duodenal administration of essential aminoacids (Low-Beer 1970, Braganza 1971, Low-Beer 1971, Low-Beer 1974, DiMagno 1975a, Low-Beer 1975, Delamarre 1984, Maton 1985). Secretion of bile acids was also shown to be reduced and delayed in celiac disease (DiMagno 1972, Brown 1987).

The combination of rapid gastric emptying (also observed in celiac patients), and reduced enzyme and bile acid secretion may contribute to the steatorrhea caused by impaired fat absorption. Maldigestion of fat in celiac sprue is thus multifactorial, being initiated by impaired CCK secretion, prolonged by excessive dilution of meals, and perhaps aggravated by abnormal metabolism or recirculation of bile acids. Steatorrhea and malnutrition might therefore be improved by pancreatic enzyme supplements or by feeding bile acids.

PERSISTENT DECREASED PLASMA CHOLECYSTOKININ LEVELS IN CELIAC PATIENTS UNDER GLUTEN-FREE DIET: RESPECTIVE ROLES OF HISTOLOGICAL CHANGES AND NUTRIENT HYDROLYSIS

1-Introduction

Celiac disease is associated with impaired cholecystokinin (CCK) release that accounts for the diminished gallbladder contraction and pancreatic function observed in patients with a flat mucosa (DiMagno 1969, Braganza 1971, DiMagno 1972, DiMagno 1975a, Calam 1982, Adler 1988, Masclee 1991, Fraquelli 1999). Appropriate CCK extraction methods and radioimmunoassays confirmed that this was related to a defective release rather than a lack of end-organ responsiveness to cholecystokinin (Low-Beer 1971, Low-Beer 1975, Brown 1987). Gluten-free diet was also demonstrated to reverse the defective CCK release (Maton 1985, Watanabe 1995).

The mechanism by which CCK release is impaired in celiac disease is however still poorly understood. Lower intestinal cholecystokinin concentrations have been reported in celiac atrophic mucosa by means of CCK extraction and radioimmunoassay (Calam 1982, Domschke 1989). On the other hand, immunocytochemistry showed an increased number of CCK cells and of the CCK immunoreactive content of celiac mucosa rather than a decrease (Polak 1979, Sjolund 1979). Another explanation for an impaired release would be a decreased stimulation of the mucosal CCK cells due to impaired intra-duodenal hydrolysis of nutrients observed with mucosal atrophy (Hopman 1995). Celiac sprue has been defined more than 30 years ago in terms of the flat lesion, usually associated with a malabsorption syndrome that responds to a gluten-free diet. Recently, more refined descriptions of gluten-induced mucosal changes have been given that might be termed the spectrum of gluten sensitivity. At least five distinctive types of mucosal lesions can be recognised and have been called pre-infiltrative, infiltrative, hyperplastic, destructive and hypoplastic (flat) (Marsh 1992). Even in histological changes of lesser degrees, some functional abnormalities have been reported, for example increased permeability in the infiltrative type (Strobel 1984, van Elburg 1993).

The aim of our study was to evaluate the cause of impaired CCK release in a larger spectrum of lesions,

i.e. control subjects and celiac patients presenting with a destructive or infiltrative type mucosa or with a totally restored mucosa under gluten-free diet. We hypothesized that if nutrient hydrolysis was the limiting factor for CCK release, it would only be present in atrophic mucosa. On the other hand, if impairment of release would still be present in the infiltrative type of mucosa, other factors or an inhibitory role of the inflammatory infiltrate should be held responsible. Furthermore, we studied the role of hydrolysis of nutrients by comparing the effects of a polymeric or a semi-elemental liquid test meal on CCK release in controls and in the different groups of celiac patients.

2-Methods

2.1 Subjects

Twenty celiac patients (5 men and 15 women) were assessed. Diagnosis of celiac disease was made through endoscopy and duodenal biopsies (n=6). Endoscopic signs of celiac disease included loss or reduction in number of duodenal folds, scalloped duodenal folds and mosaic pattern (Mainguet 1989, Corazza 1990). Histological criteria suggestive of celiac disease were: flat mucosa with surface epithelium injured, elongated crypts with mitosis, increased number of intra-epithelial lymphocytes (IELs) and chronic inflammation in lamina propria. IEL count was performed on a Leitz microscope at a magnification of 40X on hematoxylin-eosin stained slides. The number of IELs was evaluated on 100 villous epithelial cells and expressed in percentage. A value below 25 IELs/100 villous epithelial cells was considered normal (Ferguson 1971, Crowe 1994, Hayat 2002). We also performed morphometric studies including a count of villous-crypt units, the measurement of the respective length of the villi and the crypts with determination of a villous/crypt length ratio, and the measurement of the epithelial surface per villous-crypt unit.

The celiac patients were divided into 3 groups.

Group A and B were treated patients (mean age: 40 ± 2 years) under gluten-free diet for a mean period of 7.8 ± 2.9 and 7.5 ± 3.6 years, respectively. Group A patients ($n=6$) had a totally normal mucosa with a normal IEL count. Group B represented the “infiltrative type” of mucosal changes observed in celiac disease ($n=6$). Group C patients were newly diagnosed and untreated celiac patients with villous atrophy ($n=8$).

Disaccharidase levels (lactase, maltase and sucrase) were also determined on two additional jejunal biopsies, according to a previously described method (Buts 1986).

The control group in the test meal studies consisted of 9 healthy subjects (2 men and 7 women; mean age: 35 ± 5 years) without any history of gastrointestinal, general disease or prior abdominal surgery. The Ethics Committee approved the study at the University Hospital. An informed written consent was obtained for each subject.

2.2 Test meal

The subjects fasted overnight and then ingested orally a standard test meal composed of 400 ml of a liquid polymeric diet (Sondalis Iso®, Clintec, USA) or a semi-elemental diet (Reabilan®, Clintec, USA) in a mean time of 5 minutes. These meals were gluten- and lactose-free, and provided a total of 400 Kcal (1 Kcal/ml). Protein/lipid and glucid ratios were similar: 15/35/50% for Sondalis® and 12.5/35/52.5% for Reabilan®. Amino acid content also was similar for the two test meals (Table 9). Sondalis® was composed of 3.8g/100ml of protein, 3.9g/100 ml of lipids and 12.5g/100 ml of glucids (100% dextrin- maltose). Reabilan® was composed of 3.2g/100 ml of proteins, 3.5g/100 ml of lipids and 13.2 g/100 ml of glucids. Proteins were provided as a hydrolysate of lactalbumin and casein via trypsin and chymotrypsin containing 30% free amino acids, 40% small peptides of 2-4 amino acids, 23% small peptides of 5-9 amino acids and 7% of peptides with more than 9 amino acids in the semi-ele-

Table 9

PROTEIN AND AMINO ACID COMPOSITION OF THE 2 TEST MEALS: SONDALIS ISO® POLYMERIC LIQUID DIET, AND REABILAN® SEMI-ELEMENTAL LIQUID DIET, (CLINTEC, USA).

	Polymeric	Semi-elemental
Calories (kcal/100 ml)	100	100
Protein (g/100 ml)	3.8	3.2
Amino acids (%)		
Alanine	3.1	3.6
Arginine	6.0	3.3
Aspartic acid	8.7	8.4
Cysteine	0.5	1.0
Glutamine	22.4	15.9
Glycine	2.7	1.8
Histidine	3.1	2.5
Isoleucine	4.3	5.1
Leucine	7.0	10.1
Lysine	7.4	8.5
Methionine	1.4	2.3
Phenylalanine	5.2	4.6
Proline	8.5	8.4
Serine	5.3	4.7
Threonine	3.9	4.4
Tryptophane	1.3	1.8
Tyrosine	3.4	4.9
Valine	5.7	5.9

mental diet. Lipids were provided as soya and onagre oil with similar proportions of linoleic acid (11.3%), alpha-linolenic acid (0.5%) and gamma-linolenic acid (0.7%). Vitamin and mineral compositions were identical in the two test meals. Both diets were iso-osmolar (300 mOsmol/l). There were both given orally and were well tolerated by patients and volunteers, despite their marketed naso-gastric or naso-jejunal administration.

2.3 Sampling and extraction of cholecystokinin

Blood samples, 8 ml in duplicates, were drawn through an indwelling venous catheter at -15, 0, 15, 30, 45, 60, 75, 90, 105 and 120 minutes, collected on ice in glass tubes containing 2 ml of sodium acetate buffer at pH=3.6. They were immediately centrifuged at 3000 rpm at 4 °C for 12 minutes. Plasma was then diluted with 2% trifluoroacetic acid (1:3 vol/vol) and stored at 4 °C. Extraction and concentration of CCK were immediately performed by adsorption on Sep-Pak C18 cartridges (Waters Millipore, Harrows, UK) previously washed with 10 ml of acetonitrile, 10 ml of water and 10 ml of TFA 0.1% (Playford 1993). CCK was eluted with 3 ml of acetonitrile-TFA 0.1% (1:1, vol/vol) into a polypropylene vial. Eluates were lyophilised and stored at -20 °C for 1-4 weeks before radioimmunoassay. Mean recoveries of synthetic sulphated CCK-8 and CCK-33 (40 fmol/ml blood) added to whole blood prior to concentration of plasma peptides were 84-89%.

2.4 Cholecystokinin radioimmunoassay

Plasma CCK concentrations were measured by a sensitive and specific radioimmunoassay using antiserum Dino-7 as previously described (Beardshall 1992, Jordinson 1996). Dino-7 antiserum was used at a final titre of 1:106 with ¹²⁵I-Bolton-Hunter-labelled CCK-8 (Amersham, UK) as tracer, and CCK-8 as standard (Sigma, Dorset, UK). It had similar affinities for all forms of sulphated CCK (CCK-8, CCK-33, CCK-39, CCK-58). Affinity was 3000 fold lower for sulphated and non-sulphated gastrin-17. The intra- and inter-assay variability of the assay was 6.2 and 12.1%, respectively. The detection limit of the assay was 0.2 pmol/L.

2.5 Gallbladder volume measurement

Two longitudinal and two transverse images of the gallbladder were obtained by real-time ultrasonography (Toshiba SSA270A, Toshiba Corporation, Japan) using a 3.75-MHz transducer. Gallbladder volumes were calculated from these images by the sum of

cylinders (Dodds 1985). Gallbladder contraction was expressed as percent decrease of gallbladder volume before and 90 minutes after the ingestion of the test meal. Measures were performed on 6 control subjects, 6 patients of group B and 6 patients of group C.

2.6 Statistical analysis

Data were analysed on a logarithmic scale and reported as geometric means and 95% CI. Plasma CCK levels were expressed as CCK immunoreactivity in pmole/L. Integrated plasma CCK immunoreactivity was determined by calculating the area under the plasma concentration time curve after subtraction of the basal value. Groups were compared using ANOVA with F-tests and post-hoc tests were performed when significant, using the Bonferroni method. A p value of less than 0.05 was considered as statistically significant.

Table 10

CELIAC PATIENT CHARACTERISTICS: GROUP A REFERS TO PATIENTS UNDER GLUTEN FREE DIET WITHOUT INFILTRATIVE OR DESTRUCTIVE HISTOLOGICAL LESIONS, GROUP B TO PATIENTS UNDER GLUTEN FREE DIET WITH AN INFILTRATIVE PATTERN AND GROUP C TO PATIENTS WITH ATROPHIC MUCOSA.

	Group A	Group B	Group C	F-test P value
Patients (n)	6	6	8	
Age (y)	45 [35-55]	42 [36-48]	37 [32-42]	0.181
BMI (kg m ⁻²)	23 [20-26] [#]	24 [21-27] ^{**}	20 [19-21]	0.005
Flat destructive mucosa	Absent	Absent	Present	Nd
Villous/crypt length ratio	2.5 [1.8-3.1] [#]	2.1 [1.7-3.0]	0.7 [0.4-1.2]	0.01
Ep. Surf. (mm ²) /villous-crypt unit	0.037 [0.032-0.043] [#]	0.040 [0.029-0.051]	0.029 [0.021-0.038]	0.2
IELs count (n/100 enteroc.)	21 [18-25] ^{*, #}	35 [34-36]	45 [35-57]	0.0001
Lactase (IU)	9 [1-42]	20 [10-42] ^{**}	1 [0-1]	0.05
Maltase (IU)	255 [128-505] [#]	288 [192-433] ^{**}	27 [16-47]	0.0001
Sucrase (IU)	68 [34-134] [*]	63 [38-103] ^{**}	3 [1-8]	0.0001
Carotene (μmol/L)	3.1 [2.3-4.2] [#]	1.7 [0.7-3.7] ^{**}	0.5 [0.2-1.1]	0.001
Vitamin D (μmol/L)	129 [74-223] [#]	82 [63-106]	39 [22-72]	0.004
Iron (μmol/L)	20.9 [17.8-24.6] [#]	13.2 [12.4-14]	5.9 [2.2-15.9]	0.02
Endomysium antibodies	Positive 0/6	Positive 3/6	Positive 7/8	nd

NB: Groups were compared using one-way ANOVA. Post-hoc tests were performed when significant ($p < 0.05$), using the Bonferroni method. Comparison between groups: ^{*} group A vs B; [#] group A vs C; ^{**} group B vs C.

3-Results

3.1 Patients data

Patients age, body mass index (BMI), biological and histological findings are shown in **Table 10**. Typical endoscopic features of disease, atrophic mucosa and endomysium antibodies were characteristic of the untreated group of patients (group C). These patients had a significantly lower BMI ($p < 0.05$) and lower levels of lactase, sucrase and maltase in their mucosal biopsies, reflecting the atrophy and loss of disaccharidases in the brush border. Significantly lower serum concentration of beta-carotene, iron and vitamin D compared with those of group A and B patients were also observed, reflecting their malabsorption. The villous/crypt length ratio was 0.7 [0.4-1.2] compared to 2.5 [1.8-3.1] in the Group A, confirming the significantly altered duodenal mucosal morphology, with loss of villi and elongation of crypts ($p < 0.01$). The epithelial surface per villous crypt unit (0.029 mm² [0.021-0.038]) was significantly decreased compared to group A control patients (0.037 mm² [0.032-0.043], $p < 0.05$).

Patients of group A (celiac patients on a gluten-free diet with a fully normal duodenal mucosa) and group

B (celiac patients on a gluten-free diet with a duodenal mucosa characterised by a normal architecture but an inflammatory infiltrate) had similar general characteristics. The morphometric studies confirmed that the mucosal architecture in group B was normal: the villous/crypt ratio was comparable to controls as well as the epithelial surface per villous-crypt unit. The only significant histological difference observed between group A and group B was a significantly higher number of IELs ($p < 0.05$) in group B. The presence of endomysium antibodies was observed in 3/6 patients at a low level. Although lower serum iron, carotene and vitamin D levels were seen in group B these values remained in the normal ranges of values and they were not significantly different than those of patients from group A. Moreover, the disaccharidase levels and particularly the lactase level were not different between the 2 groups, confirming the absence of enterocyte brush border abnormality in both groups.

3.2 Basal CCK immunoreactivity

A significant decrease in basal CCK plasma was observed in group C celiac patients with a flat mucosa (0.6 [0.3-1.3] pmol/L) and in group B patients with the infiltrative type (0.4 [0.2-0.7] pmol/L), compared with the patients with a totally normalised mucosa

Table 11

PLASMA CCK CONCENTRATIONS BEFORE AND AFTER ADMINISTRATION OF A POLYMERIC AND SEMI-ELEMENTAL TEST MEAL IN CELIAC PATIENTS. GROUP A REFERS TO PATIENTS UNDER GLUTEN FREE DIET WITHOUT INFILTRATIVE OR DESTRUCTIVE HISTOLOGICAL LESIONS, GROUP B TO PATIENTS UNDER GLUTEN FREE DIET WITH AN INFILTRATIVE PATTERN AND GROUP C TO PATIENTS WITH ATROPHIC MUCOSA.

	Controls n=9	Group A n=6	Group B n=6	Group C n=8	F-test P value
Basal CCK (pmol/L)	1.0 [0.7-1.4]	1.5 [1.0-2.4] *	0.5 [0.3-0.7]	0.8 [0.4-1.3]	0.003
Polymeric meal					
Integrated output (pmol/L*120min)	186 [131-264]	267 [172-414] * #	56 [31-101]	34 [19-61]	0.0001
Time to peak (min)	25 [20-32]	21 [14-31] #	32 [13-79]	55 [37-82]	0.004
[Plasma CCK] Peak (pmol/L)	3.5 [2.3-5.3]	5.9 [3.0-11.5] * #	1.5 [1.1-2.1]	1.5 [1.1-2.2]	0.0001
[Plasma CCK] at 60 min (pmol/L)	2.8 [1.3-4.3]	3.5 [1.2-5.8] *	0.9 [0.5-1.3]	1.0 [0.6-1.4]	0.005
[Plasma CCK] at 120 min (pmol/L)	2.2 [1.7-2.6]	2.8 [1.1-6.6] * #	0.9 [0.7-1.2]	0.8 [0.6-1.2]	0.001
Semi-elemental meal					
Integrated output (pmol/L*120min)	201 [142-286]	224 [152-331] * #	54 [29-100]	39 [16-93]	0.002
Time to peak (min)	24 [18-34]	36 [15-39]	32 [17-62]	69 [57-82]	0.001
[Plasma CCK] Peak (pmol/L)	5.0 [2.3-6.8]	5.8 [3.3-10.1] * #	1.2 [0.7-2.3]	1.2 [0.9-1.5]	0.0001
[Plasma CCK] at 60 min (pmol/L)	3.5 [2.1-4.9]	4.1 [2.4-5.8] *	1.0 [0.5-1.5]	1.4 [0.9-1.8]	0.003
[Plasma CCK] at 120 min (pmol/L)	2.4 [1.3-3.5]	3.3 [1.6-5.0] * #	0.8 [0.4-1.1]	0.9 [0.5-1.3]	0.0001

NB: Groups were compared using one-way ANOVA. Post-hoc tests were performed when significant ($p < 0.05$), using the Bonferroni method. Comparison between groups: * group A vs B; # group A vs C. No significant difference was found between controls and group A, and between groups B and C.

(1.6 [1.0-2.4] pmol/L) and with the volunteers (1.0 [0.7-1.4] pmol/L) ($p < 0.003$) (Table 11).

3.3 Postprandial plasma CCK levels

Administration of the two test meals induced comparable integrated plasma CCK increases in all groups studied (group A-B-C and controls), as shown in Figure 5. Although no differences could be seen between the two test meals, a significantly decreased CCK release was observed in group B and C patients ($p < 0.05$). Administration of a semi-elemental diet (Reabilan®) neither corrected the defective CCK release in group B and C, nor had any effect on postprandial CCK peak values or integrated output in control subjects or in celiac patients (Table 11).

Group A patients showed normal CCK responses to both test meals. Their values were similar to those of the volunteers. Peak values of CCK immunoreactivity were significantly decreased in celiac patients with a

flat mucosa (group C) and the infiltrative type of mucosa (group B) ($p < 0.0001$) (Figure 6). This was observed with the polymeric and the semi-elemental diets.

3.4 Gallbladder volumes

Initial fasting gallbladder volumes were 19 ± 2 cm³, 23 ± 3 cm³, and 37 ± 6 cm³ in control subjects, group B and group C patients respectively. Group C fasting gallbladder volumes were significantly higher compared with the control subjects ($p < 0.05$). Significant contraction of gallbladder was observed in all groups except in group C patients with a destructive mucosa. Postprandial values were 8 ± 1 cm³ in control subjects ($p < 0.001$, compared with fasting volumes), 12 ± 1 cm³ in group B ($p < 0.005$) and 24 ± 4 cm³ (NS) in group C. No difference between the administration of the polymeric or the semi-elemental diet could be seen for gallbladder volumes and contraction in control subjects and celiac patients.

Figure 5

AUC of plasma CCK in control subjects, celiac patients under gluten free diet with a normalised mucosa (group A), with a residual IEL infiltration (group B) and untreated celiac patients (group C) after administration of a polymeric (Sondalis®) and a semi-elemental diet (Reabilan®). A significantly defective release is observed in groups B and C with the 2 test meals. Data are mean $\pm$ SEM.

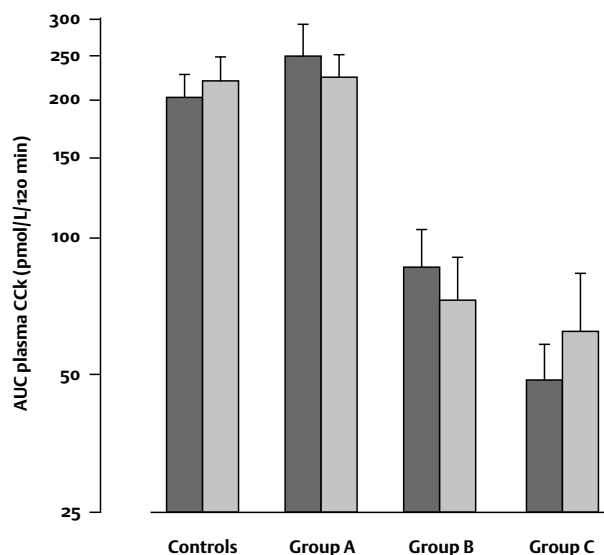
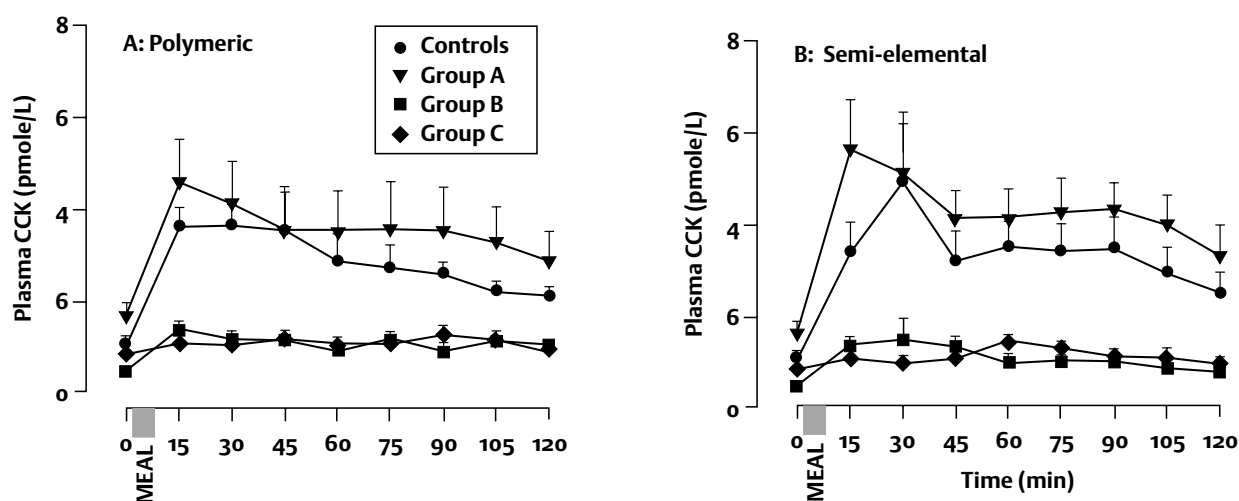


Figure 6

Meal stimulated plasma CCK release after administration of a polymeric (Sondalis® upper panel) and a semi-elemental diet (Reabilan® lower panel) in control subjects, celiac patients under gluten free diet with a normalised mucosa (group A), with a residual IEL infiltration (group B) and untreated celiac patients (group C). A significant defective CCK release is seen in group B and C with the 2 test meals. Data are mean $\pm$ SEM



4-Discussion

Our study confirmed that CCK release is impaired in untreated celiac patients. Although previous reports demonstrated a recovery of normal CCK release in patients under a gluten-free diet, we were able to show that this recovery needs full restoration of the celiac duodenal mucosa. A defective release was indeed still present in patients whose mucosa displayed a significant IEL infiltrate. Only patients without any atrophy or subatrophy and without any increased IEL infiltrate had a basal and postprandial CCK release similar to the control group. These results favour the hypothesis of an interaction between a IELs and CCK cells.

In recent years, it became apparent that gluten sensitivity is associated with a spectrum of mucosal lesions, arbitrarily termed pre-infiltrative, infiltrative-hyperplastic, flat destructive and atrophic-hypoplastic (Marsh 1992, Marsh 1995). These lesions comprise a dynamically interrelated series of events, culminating in the severe flat-destructive lesion. During treatment with a gluten-free diet, we observed in many of our celiac patients (Deprez et al, unpublished data), that the mucosa, although recovering normal architectural features, showed a persistent IEL infiltration. A persistent IEL population had already been described in children after gluten elimination and in adults with an otherwise normal mucosa (Leigh 1985, Verkasalo 1990). IEL counts in our group of patients with the infiltrative type of mucosal lesion ranged from 31 to 37 IELs/100 enterocytes. This infiltration could reflect some low but persistent intake of gluten since gluten is known to evoke a dose-responsive infiltration of IELs into the epithelium (Leigh 1985, Verkasalo 1990, Marsh 1992, Mahadeva 2002). A low intake of gluten may be suspected in our patients with the infiltrative type. Some of them have indeed a low titer of anti-endomysial antibodies.

This could allude to gluten free diet that is not optimal and that could be further improved. The patients selected to participate in this study were however compliant to their gluten free diet and they had received a fully comprehensive dietary counselling both by an expert dietician and dedicated medical staff. The persistence of IELs in their mucosa is therefore probably due to a higher sensitivity towards a very low gluten exposition. It is indeed known that a "zero level" gluten free diet is difficult to achieve since gluten free products are products containing less than 20 mg of gluten per 100gr product (European labelling). Could patchy atrophy still be present in these patients with a high IEL infiltrate and influence our results? We did not detect atrophy or

subatrophy in any of the six biopsies taken from each patient at different sites below and above the angle of Treitz. Moreover, morphometric results and disaccharidases levels were within normal values and comparable to those of our control celiac patients (group A). A recent study provides some more evidence that a raised IEL count can occur without any mucosal damage or atrophy (Leigh 1985).

However, little is known of the functions of IELs and their impact on enterocytes and mucosal endocrine cells. Their localization in celiac disease matches that of the CCK mucosal cells located in highest numbers in the duodenum and proximal jejunum and scattered evenly in the villi and crypts. Our results could therefore support some inhibitory effect of this epithelial infiltration on CCK secretion. It is tempting to speculate about the activity and interaction of duodenal cytokines on CCK cells. The cytokine profile observed in celiac disease has been approached through various experimental protocols. In patients with active disease, increased expression of interleukin (IL)-2, interferon (IFN)- γ , tumour necrosis factor (TNF)- α and TNF- β , IL-10, IL-1 β , and transforming growth factor (TGF)- β has been demonstrated in the intestinal mucosa. Thus, the expression of both Th1 (IFN- γ , IL-2) and Th2 (IL-4, IL-10) cytokines was seen in patients with active disease, implying activation of Th0 cells (Lahat 1999). Up-regulated expression of migration inhibition factor (MIF) and TNF- α in IELs and epithelial cells of histological normal mucosa was also shown in patients with celiac disease on a gluten free diet (O'Keefe 2001). Increased densities of TNF- α and IFN- γ positive cells were recently demonstrated in patients with latent disease (Westerholm-Ormio 2002). Increased expression of proinflammatory cytokines in cells occupying the epithelial layer could therefore interact with endocrine cells.

Although there is clear evidence of a communication between the immune and neuroendocrine systems, very few direct interactions have been studied in the digestive area (Beales 1997, Prinz 1997, Beales 1998). Of the putative cytokines involved in the interaction between immune and neuroendocrine systems, IL-1 and TNF- α showed inhibitory actions on gastrin and somatostatin release (Beales 1998). Raised pro-inflammatory cytokines detected by immunohistochemistry and molecular biology could provide interesting clues on such link in celiac disease (Przemioslo 1994, Kontakou 1995, Beckett 1996, Wassen 1996).

It was hypothesized that the atrophic changes of the mucosa and the loss of enteroendocrine cells in the upper small intestine could be responsible for the defective CCK release in celiac disease (Liddle 2000). Most studies reporting on CCK secretion in celiac patients have been able to show a marked decrease in plasma CCK release after stimulation with various

nutrients (Calam 1982, Hopman 1985, Maton 1985, Jansen 1992). Those studies always compared untreated patients with the typical "flat-atrophic" mucosa and patients under gluten-free diet with normalised architectural features of the jejunal mucosa. The decreased CCK secretion was shown to correlate with low intestinal CCK concentrations in jejunal extracts (Calam 1982, Domschke 1989). These observations in celiac patients were however in contrast with earlier immunocytochemical findings of increased number of CCK producing cells in celiac duodenal biopsy specimens (Polak 1979, Sjolund 1979). If we try to combine these observations, this would mean that CCK cells, even present in higher numbers, have a lower CCK content and are less likely to be stimulated by food. Absence of stimulation could be related either to impaired stimulation by the nutrients or to an impaired production of CCK despite appropriate stimulation (due to some inhibitory factors). The secretory capacity of CCK cells was preserved in celiac patients with a flat mucosa after stimulation by intravenously administered bombesin (Thimister 1998a). Maximal intravenous bombesin stimulation should however not be compared with physiological administration of nutrients but it indicated that stimulation of CCK cells from the apical side was maintained.

Other studies have suggested that maldigestion of nutrients contributed to the impaired postprandial release of CCK. CCK cells in celiac patients with a flat jejunal mucosa could indeed be stimulated when pre-digested nutrients were administered intraduodenally (Hopman 1995, Thimister 1996). This was demonstrated with an interesting though complex experimental procedure with bile and pancreatic aspiration, co-incubation with corn oil and intraduodenal re-infusion of the hydrolysed oil. This study focused on fat hydrolysis and re-infused a mixture of oil, fatty acids and components of bile and pancreatic juice. Bile and pancreatic juice could however have some inhibitory or stimulatory effects on their own, related to putative CCK releasing peptide (Green 1994, Liddle 1995, Spannagel 1998).

The second part of our study therefore aimed at evaluating the effect of nutrient hydrolysis on CCK release in a control group and in the 3 groups of celiac patients presenting either a flat mucosa, an infiltrative type lesion or a completely restored mucosa. If insufficient food hydrolysis was indeed responsible for the defective CCK release, then this would have been observed only in patients with atrophic changes and this would be partly or totally normalised with a pre-digested test meal. We compared two orally administered liquid standard meals, one polymeric with intact lactalbumin and casein and the other semi-elemental with protein hydrolysis and a high proportion of small peptides. The semi-elemental diet comprised more than 30% amino acids and 63% of small peptides of less than 9 amino acids. These test meals were cho-

sen because they had similar amino-acid composition. Gastric emptying rates of the polymeric and semi-elemental types were measured and were shown to be normal (data not shown) and similar in all groups. Normal gastric emptying rate would have minimized protein hydrolysis of the liquid meal in the stomach itself.

CCK release with the polymeric or semi-elemental diets was similarly decreased in patients with an atrophic mucosa. This significant decrease, compared with the control group and the celiac patients with a completely normalised mucosa, was observed both for the plasma CCK peak and the integrated output. This demonstrated the ineffectiveness of protein hydrolysis to correct the defective CCK release observed in celiac patients with a flat mucosa during the 2 hours after ingestion of the test meal. Furthermore a similar defective release of CCK was shown in the group of patients with the infiltrative mucosal pattern. This was observed with the 2 test meals, indicating again that pre-digestion of food did not correct the defective release of CCK. This would support an identical defective mechanism in celiac patients with atrophy or an infiltrative type mucosa. Atrophy *per se* would not be the cause of the defective CCK release. Normal and similar postprandial CCK secretion was observed with both test meals in control subjects and celiac patients with a normalised mucosa. End-organ stimulation, examined through postprandial gallbladder contraction, was diminished in our celiac patients and was not increased by protein pre-digestion.

Protein and fat are the major constituents of a meal that stimulates CCK release, pancreatic exocrine secretion, and gallbladder contraction (Ivy 1928, Wiener 1981, Hopman 1985, Fried 1988, Fried 1989). Release of CCK during nutrient administration is however species specific. In rats, only intact proteins, but not amino acids, stimulate the release of CCK and pancreatic exocrine secretion (Green 1983, Liddle 1986). In dogs, amino acids were found to be more effective than intact proteins in stimulating pancreatic exocrine secretion (Meyer 1976). Digestion of protein has also been shown to be of crucial importance for stimulation of CCK release, gallbladder contraction and pancreatic enzyme secretion in men (Thimister 1996). Data in men is however somewhat contradictory, since other reports failed to demonstrate a significant difference in CCK release when intact proteins and amino acids were compared or when proteases were added to the test meals (Malagelada 1976, Liddle 1985, Slaff 1985, Owyang 1986a, Liener 1988).

Intraduodenal proteolytic and lipolytic activities have also been studied and lipolysis was shown to be important for the stimulation of CCK release in healthy volunteers and patients with pancreatic insufficiency (Guimbaud 1997, Hildebrand 1998).

The discrepancy observed between these studies and our results could be related to differences in the respective effects of proteins and fat on CCK secretion, the latter being more dependent on pre-digestion and solubilisation of lipolytic products. Micellar solubilisation of fatty acids and monoglycerides could be an important step for delivery of lipolytic products to the aqueous barrier of the epithelial and endocrine cells. The hypothesis that this could be due to elongated crypts and a more difficult access of nutrients to the CCK cells is however not confirmed by our results showing a persistent decrease of CCK secretion in patients with normal architectural features but persistent IEL infiltration. The significantly lower pre-prandial values of CCK in celiac patients with atrophic and infiltrative mucosal changes compared with control subjects and normal celiac patients under gluten-free diet is also in favour of a local inhibitory mechanism. Whether this local

inhibitory mechanism is affecting the CCK cells directly or exerting an action on the newly discovered luminal CCK-releasing factor is unknown (Wang 2002).

In conclusion, we demonstrated that the intraepithelial lymphocytic infiltrate observed in the intestinal mucosa of patients with celiac disease is associated with decreased plasma CCK levels. CCK post-prandial plasma secretion was not improved by the administration of a pre-digested standard meal. Impaired CCK release in celiac disease is therefore not due to reduced protein hydrolysis in the duodenal lumen that was previously linked to mucosal atrophy. Some inhibitory mechanism could be involved in the CCK cell dysfunction observed in celiac patients presenting with lesser degrees of disease activity. Further studies should focus on the relationship between IELs and endocrine mucosal cells.



EXPRESSION OF CHOLECYSTOKININ IN THE DUODENUM OF PATIENTS WITH CELIAC DISEASE: RESPECTIVE ROLE OF ATROPHY AND LYMPHOCYTIC INFILTRATION.

1-Introduction

Celiac disease is associated with reduced circulating concentration of cholecystokinin (CCK) that accounts for the diminished gallbladder contraction and pancreatic function observed in patients with a flat mucosa (Dreiling 1957, DiMagno 1969, Braganza 1971, DiMagno 1972, DiMagno 1975a, Regan 1980, Calam 1982, Fraquelli 1999). Appropriate CCK extraction methods and radioimmunoassays confirmed that this was related to a defective release rather than a lack of end-organ responsiveness to cholecystokinin (Low-Beer 1971, Low-Beer 1975, Brown 1987, Masclee 1991). Gluten-free diet was also demonstrated to reverse the defective CCK release (Maton 1985). The mechanism by which CCK release is impaired in celiac disease is however still poorly understood. Lower intestinal CCK concentrations have been reported in celiac atrophic mucosa by means of CCK extraction and radioimmunoassay (Calam 1982, Domschke 1989). These observations are in opposition with immunocytochemistry reports showing an increased number of CCK cells in the celiac mucosa (Polak 1979, Sjolund 1979). Impaired release could also be explained by a decreased stimulation of the mucosal CCK cells due to impaired intra-duodenal hydrolysis of nutrients observed with mucosal atrophy (Hopman 1995).

Celiac sprue has been defined more than 30 years ago in terms of the flat lesion, usually associated with a malabsorption syndrome that responds to a gluten-free diet (DiMagno 1969, DiMagno 1972). Recently, more refined descriptions of gluten-induced mucosal changes have been given that might be termed the spectrum of gluten sensitivity. At least five distinctive types of mucosal lesions can be recognised and have been called pre-infiltrative, infiltrative, hyperplastic, destructive and hypoplastic (flat) (Marsh 1992). Even in histological changes of lesser degrees, some functional abnormalities have been reported, for example increased permeability (van Elburg 1993, Cummins 2001). The aim of our work was to determine the origin of CCK deficiency and to study the relationship between the distinctive mucosal patterns observed in

celiac disease and the mucosal CCK content and specifically to determine if CCK deficiency was due to a decrease in the number of CCK cells, a decrease in peptide concentration or CCK mRNA content.

2-Methods

2.1 Patients

Biopsies (n=6) were obtained from the distal duodenum, just proximal to the angle of Treitz, during upper GI endoscopy in 10 controls and 19 celiac patients (5 men, 14 women). Celiac patients were divided in three groups: untreated patients presenting with atrophic mucosa [group A; n=7], patients on a gluten-free diet with intraepithelial lymphocytic infiltration but no architectural changes of the mucosa [group B; n=6] and patients on a gluten-free diet with a fully normalised mucosa [group C; n=6]. Diagnosis of celiac disease was made using jejunoscopy and jejunal biopsies. Endoscopic signs of celiac disease included loss or reduced number of duodenal folds, scalloped duodenal folds and mosaic pattern (Mainguet 1989, Corazza 1990).

Histological criteria suggestive of celiac disease were: flat mucosa with injured surface epithelium, elongated crypts with mitosis, increased number of intraepithelial lymphocytes (IELs) and chronic inflammation in lamina propria [18]. IEL count was performed on a Leitz microscope at a magnification of 40X on hematoxylin-eosin stained slides. The number of IELs was evaluated on 100 villous epithelial cells and expressed as percentage. A value below 25 IELs/100 villous epithelial cells was considered normal (Ferguson 1971, Dobbins 1986, Crowe 1994). We also performed morphometric studies including a count of villous-crypt units, the measurement of the respective length of the villi and the crypts with determination of a villous/crypt length ratio, and the measurement of the epithelial surface per villous-crypt unit.

Control subjects (3 men, 7 women) underwent upper gastrointestinal endoscopy for evaluation of dyspeptic complaints; their gastric or duodenal mucosa showed a normal endoscopic appearance and no histological changes. They were matched for age (41 ± 4 years) with celiac patients. The research was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association, and was approved by the Ethics Committee of the Faculty of Medicine. An informed written consent was obtained for each subject or patient.

2.2 Immunocytochemistry

A double immunocytochemical staining to specifically recognise the CCK-cells was performed on paraffin-embedded fixed specimen. A polyclonal antibody raised against the N-terminal gastrin-34 (LW21) was used at a 1:4000 dilution, and revealed by streptavidin-biotin-peroxydase system and DAB (brown staining) [26]. After elution, the same slides were incubated overnight with a polyclonal antibody raised against the C-terminal CCK-10 (Dino) at a 1:10000 dilution and revealed by an alkaline phosphatase antialkaline phosphatase system (red staining) (Playford 1993, Jordinson 1996). This antibody, although highly specific for CCK in radioimmunoassays, had a low affinity for gastrin cells in immunocytochemistry. After exclusion of the brown stained cells, the red stained cells were considered to be specifically CCK cells and were counted. The number of CCK-positive cells was determined per villous-crypt unit (Buchan 1984). Their number was also referred to the epithelial area of each biopsy. This epithelial area was measured on adjacent tissue sections immunostained in brown with an antihuman cytokeratin monoclonal antibody (Clone CAM 5-2; 1/25) (Becton Dickinson, Erembodegem, Belgium) using a semi-automatic image analyser (IBAS 2000-Kontron, Munich, FRG) (Demarchi 2003).

2.3 Tissue extraction and radioimmunoassay

CCK was extracted from intestinal biopsies in acid (TFA 2%) and boiling water (1 biopsy in 2 ml) for 5 minutes. After homogenisation and centrifugation (10 min, 10000 rpm), the supernatant was decanted. Further extraction and concentration of CCK were immediately performed by adsorption on Sep-Pak C18 cartridges (Waters Millipore, Harrows, UK) previously washed with 10 ml of acetonitrile, 10 ml of water and 10 ml of 0.1% TFA (Playford 1993). CCK was eluted with 3 ml of acetonitrile in 0.1% TFA (1:1, vol/vol) into a polypropylene vial. Eluates were lyophilised and stored at -20°C for 1-4 weeks before performing radioimmunoassay. Plasma CCK concen-

trations were measured with a sensitive and specific radioimmunoassay using Dino-7 antiserum as previously described (Jordinson 1996). Dino-7 antiserum was used at a final titre of 1:106 with ^{125}I -Bolton-Hunter-labeled CCK-8 (Amersham, UK) as a tracer and CCK-8 as a standard (Sigma, Dorset, UK). It had similar affinities for all forms of sulphated CCK (CCK-8, CCK-33, CCK-39, CCK-58). Affinity was 3000 fold lower for sulphated and non-sulphated gastrin-17. The intra- and inter-assay variability of the assay was 6.2 and 12.1%, respectively. The detection limit of the assay was 0.2 pmol/L.

2.4 Semiquantitative RT-PCR

Total RNA was prepared from jejunal biopsies (47-195 mg) using the guanidine thiocyanate and caesium chloride method (Chomczynski 1987). Five micrograms of jejunal RNA were preincubated with random hexamer and water for 10 min at 70°C . Four hundred units of Moloney murine leukaemia virus reverse transcriptase (Gibco BRL, Merelbeke, Belgium) were added together with dTT (dithiothreitol) and dNTPs (deoxynucleoside triphosphate), and the reaction was continued for further 60 minutes at 37°C . PCR was carried out with primers specific for human CCK and ribosomal protein L19 (RPL19) as an internal standard (Colin 1995). PCR was performed in two steps to ensure that both CCK (5 cycles) and RPL19 (25 cycles) be in the linear range for amplification. The first 5-cycle PCR step (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) was performed using only the CCK primers in 30 μl of a solution containing 3 μl of PCR buffer, 30 pmoles each of 5'- and 3'-CCK primers, 6.4 μl of dNTPs, 0.4 μl of [^{32}P] deoxyCTP (1.25 μCi ; Amersham, Essex, UK) and 1.25 U of Taq polymerase (Boehringer Mannheim, Brussels, Belgium). The reaction was kept at 25°C and followed by a second 25-cycle PCR (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) after the addition of 50 μl of a solution containing 1 X PCR buffer, 30 pmoles each of 5'- and 3' RPL19 primers, 1.1 mM of MgCl_2 , and 1.25 U of Taq polymerase. DNA contamination of RNA was excluded by the absence of product in the reaction performed without RT.

The following primers were used: CCK sense: GTATCGCAGAGAACGGAT, antisense TACTCAT-ACTCCTCGGCACT (size of product: 203bp), RPL19 sense AGTATGCTCAGGCTTCAGAA; antisense TTCCTTGGTCTTAGACCTGC (size of the product: 501 bp). PCR products were separated using 1.5% agarose gel electrophoresis. The gels were vacuum-dried (LKB 2003, Slab gel dryer, Sweden) and exposed to x-ray film (Kodak Biomax MS, Boston USA) for 1 h at room temperature. The PCR bands were quantified by computer-assisted densitometric scanning (Ultrosan XL LKB bromma and Gelscan PC). Individual PCR reactions were calculated as a ratio of

CCK to RPL19 and the ratio was averaged across 4 dilutions (1/1; 1/2.5; 1/5; 1/10) from each sample [30]. Experiments were performed in duplicates.

2.5 Statistical analysis

Data were analysed on a logarithmic scale and reported as geometric means and 95% CI. The number of CCK cells was expressed as a ratio of cells to square millimetre of epithelial area; jejunal extracts CCK concentrations as CCK immunoreactivity in pmole/g of tissue and CCK-mRNA as a ratio between CCK and RPL19. Groups were compared using ANOVA with F-tests and post-hoc tests were performed when significant, using the Bonferroni method. A p value of less than 0.05 was considered as statistically significant.

3-Results

3.1 Patients

Patients' age, body mass index (BMI) and biological findings are shown in **Table 12**. Typical endoscopic features of disease, atrophic mucosa and endomysium

antibodies were characteristic of the untreated group of patients (group A). The villous/crypt length ratio was 0.7 [0.4-1.2] compared to 2.9 [2.2-3.4] in the control group, confirming the significantly altered duodenal mucosal morphology, with loss of villi and elongation of crypts ($p < 0.01$). The epithelial surface per villous crypt unit (0.029 mm^2 [0.021-0.038]) was significantly decreased compared to controls (0.048 mm^2 [0.034-0.058], $p < 0.05$). These patients had a significantly lower BMI ($p < 0.05$) and lower serum concentration of beta-carotene, iron and vitamin D as compared with patients of group B and C, which reflected malabsorption. Patients of group B (celiac patients on a gluten-free diet with a duodenal mucosa characterised by a normal architecture but an inflammatory infiltrate) and group C (celiac patients on a gluten-free diet with a fully normal duodenal mucosa) had similar general characteristics. The morphometric studies confirmed that the mucosal architecture returned to normal: the villous/crypt ratio was comparable to controls as well as the epithelial surface per villous-crypt unit (**Table 13**). The following differences were observed between group B and group C: a significantly higher number of IELs ($p < 0.05$) in group B, a low serum iron concentration and a more frequent presence of endomysium antibodies in group B.

Table 12

CELIAC PATIENT CHARACTERISTICS: GROUP A REFERS TO UNTREATED PATIENTS WITH ATROPHIC MUCOSA, GROUP B TO PATIENTS ON A GLUTEN-FREE DIET WITH AN INFILTRATIVE PATTERN AND GROUP C TO PATIENTS ON A GLUTEN-FREE DIET WITHOUT INFILTRATIVE OR DESTRUCTIVE HISTOLOGICAL LESIONS.

	Group A	Group B	Group C	P
Patients (n)	7	6	6	
Age (y)	39 [33-44]	42 [36-48]	45 [35-55]	0.181
BMI (kg m^{-2})	19 [18-20]	24 [21-27] *	23 [20-26] °	0.005
Flat destructive mucosa	Present	Absent	Absent	Nd
Villous/crypt length ratio	0.7 [0.4-1.2]	1.9 [1.5-2.3]	2.5 [1.8-3.1] °	0.01
Ep. Surf. (mm^2) /villous-crypt unit	0.029 [0.021-0.038]	0.040 [0.029-0.051]	0.037 [0.032-0.043] °	0.2
IELs count (n/100 enterocytes)	43 [33-55]	35 [34-36]	21 [18-25] ***	0.0001
Carotene ($\mu\text{mol/L}$)	0.7 [0.3-1.1]	1.7 [0.7-3.7] *	3.1 [2.3-4.2] °	0.001
Vitamin D ($\mu\text{mol/L}$)	43 [25-75]	82 [63-106]	129 [74-223] °	0.004
Iron ($\mu\text{mol/L}$)	6.3 [3.4-16.7]	13.2 [12.4-14]	20.9 [17.8-24.6] °	0.02
Endomysium antibodies	Positive 6/7	Positive 3/6	Positive 0/6	nd

NB: Results are expressed as means and 95% CI. Groups were compared using one-way ANOVA. Post-hoc tests were performed when significant ($p < 0.05$), using the Bonferroni method. Comparison between groups: * group B vs A ** group C vs B; ° group A vs C.

3.2 Immunocytochemistry

The results of cell quantification are shown in **Table 13**. CCK-positive cell count was higher than gastrin-containing cell count. CCK cell size did not change, and CCK cells were found in crypts and villi both in controls and patients with an infiltrative pattern or a normalised mucosa, and throughout the crypts of patients with atrophic mucosa (**Figure 7**). A non-significant decrease in the number of CCK cells, in the epithelial surface and in the ratio of CCK cells to epithelial surface was observed in group A (patients with atrophic mucosa).

When CCK-positive cells were expressed per villous-crypt unit, a trend towards a decreased number of CCK-positive cells was observed without reaching statistical significance. When the individual patients' characteristics were analysed, we observed some distinctive patterns: 2 patients (group A) showed an absence of CCK cells and 2 other patients (group A) showed a very high CCK cell count (40 and 67) and higher ratios (15.6 and 39.9). No relationship or correlation could be seen between these results and those from tissue extraction or RT-PCR. These discrepancies were not observed in the other groups.

3.3 Tissue extraction and radioimmunoassay

The results are shown in **Table 13**. Between-group comparison showed a significant decrease of CCK

Figure 7

Immunocytochemical staining for CCK cells in a duodenal biopsy from a celiac patient with atrophic mucosa (alkaline-phosphatase x 520).

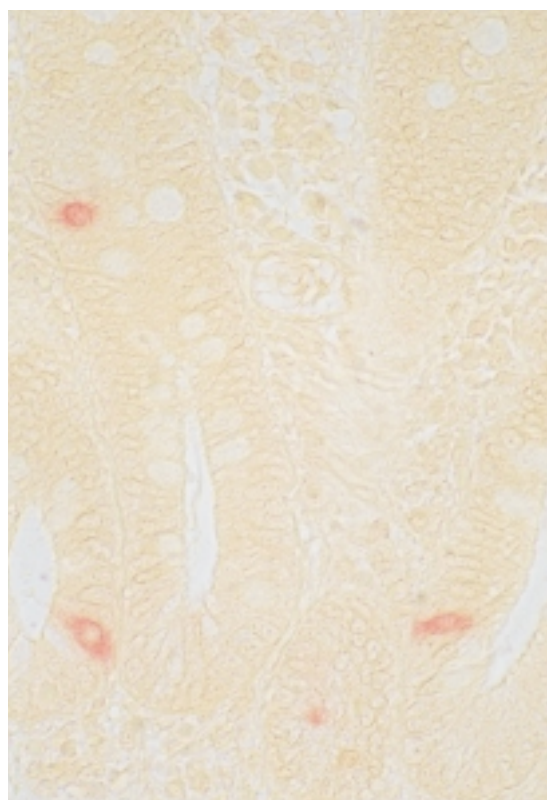


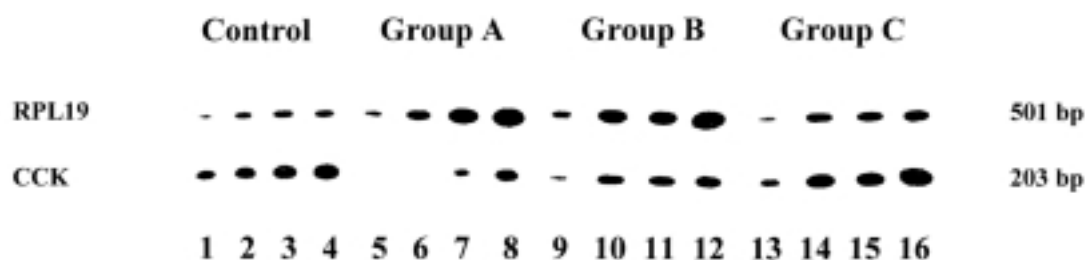
Table 13

QUANTIFICATION RESULTS: GROUP A REFERS TO UNTREATED PATIENTS WITH ATROPHY, GROUP B TO PATIENTS ON A GLUTEN-FREE DIET WITH AN INFILTRATIVE PATTERN AND GROUP C TO TREATED PATIENTS WITH A NORMALISED MUCOSA WITHOUT HISTOLOGICAL LESIONS. RESULTS ARE EXPRESSED AS MEANS AND 95% CI.

	Controls	Group A	Group B	Group C	F-test Pvalue
N	10	7	6	6	
CCK cells / surface ratio	12.1 [2.6-21.4]	8.7 [2.1-19.4]	9.3 [1.6-17.1]	10.8 [5.2-16.4]	0.96
CCK cells / villous-crypt unit	0.49 [0.19-0.63]	0.39 [0.28-0.47]	0.45 [0.32-0.56]	0.46 [0.33-0.58]	0.21
[CCK] pmol/g Neutral extracts	42.7 [18.2-67.2]	12.2 [6.9-17.5]	13.9 [3.8-31.8]	40.5 [30.4-50.7]	0.001
[CCK] pmol/g Acid extracts	12.3 [6.4-19.5]	6.4 [1.9-12.1]	7.8 [2.2-14.5]	11.3 [5.7-22.3]	0.04
CCK mRNA (CCK/RPL19 ratio)	1.35 [1.09-1.62]	0.64 [0.30-0.99]	0.86 [0.57-1.15]	1.40 [0.41-2.40]	0.03

Figure 8

Semi-quantitative assessment of CCK mRNA with a two-step RT-PCR using RPL19 as an internal standard. PCR products were separated using 1.5% agarose gel electrophoresis and exposed to X-ray film for 1 hour. Jejunal extracts were obtained from controls (Control; lanes 1-4); celiac patients with atrophic mucosa (Group A; lanes 5-8), intraepithelial lymphocytic infiltration (Group B, lanes 9-12) and completely restored jejunal mucosa (Group C, lanes 13-16). PCR results were calculated as a ratio of CCK to RPL19 and averaged across 4 dilutions (1/10, 1/5, 1/2.5, 1/1).



concentration in patients of group A with atrophic changes ($p=0.006$), but surprisingly also in patients of group B with an infiltrative pattern ($p=0.01$). Some patients in the group A with atrophic mucosa showed very low CCK concentrations. We did not observe any patients with high levels of CCK as it was observed with immunocytochemistry. In particular the two patients with high CCK cell count had low concentrations of CCK in their extracts. The patients with no CCK cells detectable by immunocytochemistry had CCK tissue concentrations comparable with the other patients in the same group.

3.4 Semiquantitative reverse RT-PCR

Results are shown in **Figure 8**. Significantly lower concentrations of CCK-mRNA were seen in patients with a destructive type mucosa as compared to controls and treated patients with a normal mucosa (CCK/RPL19 ratio, $p=0.03$). Interestingly, a similar decrease was also seen in the jejunal biopsies with an infiltrative pattern ($p=0.05$).

4-Discussion

The present studies provide evidence that the defective release of CCK observed in patients with celiac disease is not related to a decrease in the number of CCK cells present in the proximal part of the small intestine but rather to a decrease in CCK synthesis mediated by a decrease in mRNA content. Previous results in the literature were at variance in

this prospect with reports of increased CCK cell counts in celiac mucosa (Polak 1979, Sjolund 1979, Buchan 1984) and decreased amounts of CCK in jejunal extracts (Calam 1982, Domschke 1989). These discrepancies could be related to the site where the pathological specimen was taken (duodenum, Treitz angle or proximal jejunum), to methodological difficulties in counting endocrine cells in atrophic or non atrophic mucosa, to the method used to refer the positive cells on epithelial cells, to the various antisera used (recognition of smaller and larger CCK forms), to differences in the degree of mucosal lesions, or to the type of celiac patients studied (on a normal diet or a gluten-free diet). The size of the cells could also account for some discrepancy since CCK cells were shown to be more numerous but smaller in one report (Buchan 1993) and larger in another study (Polak 1979).

We studied 19 patients divided in 3 groups according to the severity of their mucosal lesion, and we could not show any significant differences in the number of CCK cells in specimens taken from the distal duodenum. A non-significant trend towards fewer CCK expressing cells was observed in our group of patients with mucosal atrophy. This trend was observed when CCK-positive cells were expressed per surface ratio and per villous-crypt unit. A similar trend was not observed however in the two other groups of celiac patients (fully recovered mucosa and mucosa with raised intraepithelial lymphocytes). Some individual differences could be seen, particularly in the group of patients with atrophic mucosa, either with an absence of CCK cells or with a larger number of cells. These results were not related to the degree of atrophy and could be explained by sampling differences since celiac disease is said to be patchy.

Decreased amounts of CCK in the duodenal mucosa of celiac patients were observed both in neutral and acidic extracts. Our CCK antiserum was able to recognise all molecular forms of CCK with a comparable affinity (Jordinson 1996). The similar decrease of CCK content in neutral and acidic extracts would confirm that all molecular forms are affected in celiac disease as previously published (Calam 1982). The decreased levels of CCK in the mucosa of patients on a gluten-free diet (for a mean duration of 3.6 years) showing persistent infiltration of IELs was more surprising. This persistent infiltrate is not uncommon in our patients (>65%, unpublished data) and has been previously reported (Verkasalo 1990). It could reflect some low but persistent intake of gluten since gluten is known to evoke a dose-responsive infiltration of IELs into the epithelium (Leigh 1985, Marsh 1992).

In recent years, it became apparent that gluten sensitivity is associated with a spectrum of mucosal lesions, arbitrarily termed pre-infiltrative, infiltrative-hyperplastic, flat destructive and atrophic-hypoplastic (Marsh 1992, Marsh 1995). These lesions comprise a dynamically interrelated series of events, culminating in the severe flat-destructive lesion. IEL counts in our group of patients with the infiltrative type of mucosal lesion ranged from 31 to 37 IELs/100 enterocytes (**Table 13**). Little is known about the functions of IELs and their impact on enterocytes and mucosal endocrine cells. Their localisation in celiac disease matches that of the CCK mucosal cells located in highest numbers in the duodenum and proximal jejunum and scattered evenly in the villi and crypts.

Our results could therefore support some inhibitory effect of this epithelial infiltration on CCK secretion. It is tempting to speculate about the activity and interaction of duodenal cytokines on CCK cells. Although there is clear evidence of a communication between the immune and neuroendocrine systems, very few direct interactions have been studied in the digestive area. Raised pro-inflammatory cytokines detected by immunohistochemistry and molecular biology could provide interesting clues on such link in celiac disease (Przemioslo 1994, Kontakou 1995, Beckett 1996, Wassen 1996).

A decreased CCK content in the jejunal mucosa could also be explained by paracrine inhibition (somatostatin) or defective stimulation by intraduodenal nutrients. Somatostatin is located in D cells throughout the gastrointestinal tract and is believed to act primarily as a paracrine factor (Yamada 1987). Celiac patients have been shown to have higher

somatostatin levels than controls and this increase was supposed to cause the higher gallbladder volumes observed in fasting celiac patients (Fraquelli 1999). Immunocytochemical studies have however reported normal or decreased somatostatin levels in celiac patients with a flat mucosa (Buchan 1984, Domschke 1989). Our data do not support a role of somatostatin in impairing CCK secretion.

Defective stimulation of CCK release has been demonstrated in celiac patients with a destructive pattern (Hopman 1995). In the presence of an atrophic mucosa, crypts are elongated and most CCK cells reside in the bottom of the crypts where contact with food would be insufficient (Buchan 1984). Moreover, administration of pre-digested fat corrected the defective CCK secretion in those patients. However, a similar mechanism has never been shown in patients with mucosal lesions limited to an inflammatory infiltrate. Those patients do not show any clinical or biological sign of malabsorption and their mucosa has no architectural modification that could lead to impaired contact of nutrients and the CCK cells. Our studies therefore suggest a more complex interaction between food, CCK release, CCK duodenal cells and the various patterns of intestinal lesions observed in celiac disease, including the intraepithelial lymphocytic infiltration.

The decrease in duodenal CCK concentrations was associated with decreased mRNA abundance, showing evidence of a coupling between hormonal secretion and gene expression. Although short-term experiments failed to demonstrate an association between CCK secretion and corresponding increases of mRNA, dietary stimulation of CCK secretion was reported to be associated with an increase in CCK mRNA levels that was due, at least in part, to an increase in CCK gene transcription (Liddle 1988, Kanayama 1991, Bentouimou 1995). A similar coupling between jejunal CCK content and mRNA levels could therefore have been expected in our results, since the patients studied were either untreated celiac patients or treated patients following a gluten-free diet for a mean of 3.6 years.

In conclusion, our results confirm an impaired upper intestinal endocrine function in celiac disease. We demonstrated a decrease in jejunal CCK concentrations and a reduction in CCK mRNA transcripts without significant changes in the number of CCK expressing cells, especially in patients with high intraepithelial lymphocytes counts. Defective CCK release could therefore be related to suppressive factors induced by the inflammatory infiltrate.

MODULATION OF CHOLECYSTOKININ RELEASE BY CYTOKINES ON SHORT-TERM CULTURED DUODENAL BIOPSY SAMPLES AND ON STC₁ CELLS.

1-Introduction

Cholecystokinin (CCK) is a peptide hormone that is released from the upper small intestine in response to food. CCK regulates many aspects of gastrointestinal function including gallbladder contraction (Liddle 1985), exocrine pancreatic secretion (Beglinger 1985), and gastric emptying (Fried 1991). Deficient release of CCK has mainly been described in celiac disease (Dreiling 1957, DiMagno 1969, DiMagno 1972, Calam 1982, Fried 1991) but also in some rare auto-immune disorders and bulimia nervosa (Geraciotti 1988). The impaired CCK release in celiac disease accounts for the diminished gallbladder contraction and pancreatic function observed in patients with a flat mucosa and participates to their malabsorption (DiMagno 1975b, Regan 1980, Brown 1987, Geraciotti 1988). Reversal of the defective cholecystokinin release and related pancreatic or gallbladder dysfunction is usually observed by gluten-free diet (Maton 1985). The persistence of diarrhoea in treated celiac sprue is however frequently seen and can be related to persistent pancreatic insufficiency. We observed in previous studies that a subset of patients under gluten-free diet still presented with an intraepithelial lymphocytic infiltrate and that this infiltrate was associated with a persistent defective release of CCK (Deprez 2002b).

We further showed that CCK defective release was not due to a decrease in the number of CCK cells but rather to diminished jejunal CCK concentrations and CCK mRNA expression (Deprez 2002a). Defective CCK release could therefore be related to suppressive factors induced by the inflammatory infiltrate. Celiac sprue has been defined more than 30 years ago in terms of the flat lesion, usually associated with a malabsorption syndrome that responds to a gluten-free diet (Marsh 1992). Recently, more refined descriptions of gluten-induced mucosal changes have been given that might be termed the spectrum of gluten sensitivity (Leigh 1985). Intraepithelial lymphocytes are increased in the early mucosal changes observed in the duodenal mucosa of celiac patients. Little is known about their function and their impact on ente-

rocytes and mucosal endocrine cells. Their localisation in celiac disease matches that of the CCK mucosal cells located in highest numbers in the duodenum and proximal jejunum and scattered evenly in the villi and crypts. It is tempting to speculate about the activity and interaction of duodenal cytokines on CCK cells.

Although there is clear evidence of a communication between the immune and neuroendocrine systems, very few direct interactions have been studied in the digestive area (Wassen 1996, Beales 1997, Prinz 1997, Beales 1998). Raised pro-inflammatory cytokines detected by immunohistochemistry and molecular biology in celiac disease could provide interesting clues on modulation of CCK release (Przemioslo 1994, Kontakou 1995, Beckett 1996, Troncone 1998, Lahat 1999, Monteleone 2001, O'Keeffe 2001). We therefore studied the effects of cytokines produced by mucosal lymphocytes on the CCK secreting murine neuroendocrine tumour cell line STC-1 and short-term organ cultures of duodenal biopsy specimens obtained from normal controls to investigate the immuno-neuroendocrine relationship.

2-Methods

2.1 Patients

Mucosal biopsy specimens were obtained by endoscopy (Olympus GIF 100) from the third or fourth part of the duodenum from 28 adult patients (20 men; median age 45, range 28-72) with upper gastrointestinal disorders, with ethics committee approval and patients inform consent. Patients had no visible abnormalities as assessed by endoscopy and histological examination of the specimens. Six to twelve specimens per patient were obtained per patient and expelled from biopsy forceps into HBSS medium at room temperature and extensively washed (three times) in 30 ml of this solution.

2.2 Chemicals

Hank's balanced salt solution (HBSS) was obtained from Bio-Whittaker, Verviers, Belgium. Fetal calf serum, RPMI 1640 with 25MM Hepes buffer and L-glutamine, Dulbecco's modified Eagle's medium (HG-DMEM) were purchased from Gibco, Merelbeke, Belgium. Falcon multiwells were from Merck Eurolab, Leuven, Belgium. Bombesin, L-phenylalanine, Tryptan blue (solution 0.4%), penicillin, streptomycin from Sigma-Aldrich, Bornem, Belgium. Purified phytohaemagglutinin was obtained from Murex Diagnostics, Chatillon, France. Recombinant human IL-2, IL-4, IL-6, IL-8, IL-10, Rantes were purchased from Immunosource, Zoersel-Halle, Belgium. Recombinant human TNF- α and IL- β were from Gemzyme Corporation, Cambridge, MA, USA. Sep-pak C18 cartridges were purchased from Waters Associates, Milford, USA. ^{125} I-Bolton-Hunter-labeled CCK-8 was from Amersham, UK. The STC-1 cell line was kindly provided by Prof JCl. Cuber (Hopital Edouard Herriot, Lyon, France).

2.3 Cell culture

The STC-1 cell line was derived from an intestinal endocrine tumour that developed in mice carrying the transgenes for the rat insulin promoter linked to the simian virus 40 large T antigen and the polyoma virus small t antigen (Chang 1994). STC-1 cells were grown in solution containing 150 ml/l horse serum, 25 ml/L foetal calf serum, 100 kU/L penicillin, and 100mg/L streptomycin in high-glucose Dulbecco's modified Eagle's medium (HG-DMEM) (Nemoz-Gaillard 1998a). Cells were passaged after gentle dissociation with 0.25% trypsin every 2-4 Cells were incubated at 37°C and aerated with a 95% air-5% CO₂ gas mixture. They were studied between passages 32 and 92, in 6-well culture dishes 5-6 days after passage when cells were 80-90% confluent, each well containing about 1 million cells. For secretory studies cells were incubated during 1 h in HBSS (Ca and Mg supplemented) with the various stimulants, cytokines, LIE and LPL supernatants.

2.4 Organ culture

Specimens were divided in 2 parts to provide explants of approximately two square mm, were weighed, placed into 24-wells sterile plastic culture Falcon dishes and incubated in 1 ml RPMI 1640 medium containing 10% foetal calf serum at 37°C in a humidified 5% carbon dioxide/95% air atmosphere for 2 hours. Supernatants were then removed and replaced with incubating media containing the tested substances for 1 hour in the same conditions. After

incubation, the supernatant was immediately processed for CCK extraction and placed on ice, homogenised during 30 sec, sonicated for 30 sec and centrifuged to remove debris at 4°C, 10 min at 10000 rpm. Hundred μ l of the supernatant were used for protein concentration measurement following Micro BCA™ Protein Assay (Pierce, Aalst, Belgium). Viability was assessed by histology, measurements of lactate deshydrogenase levels (LDH) in supernatant and biopsies and by tryptan blue exclusion (O'Brien 1970).

2.5 IEL and LPL supernatants

IEL and LPL supernatants were separately isolated from human colon mucosa and prepared by a previously published method (Hoang 1991). Median yield of IEL was 5×10^5 cells/g tissue. Contamination by epithelial cells with the IEL and LPL isolates was less than 10% (Hoang 1992a). IEL or LPMNC at a concentration of 1×10^6 were cultured in 1 ml aliquots with or without 10 μ g/ml PHA for 72 hours at 37°C and 5% CO₂. The supernatants were then harvested, centrifuged and passed through a 0.22 μ m filter and stored at -70°C for subsequent studies. Cytokine analysis into IEL and LPL supernatants showed low median concentrations of IFN- γ of 0.3 IU/ml (range 0-0.6) and 1.5 IU (range 1-8), IL-2 of 1 IU (range 0-20) and 2.1 IU (range 0.8-2.4), and IL-10 of 8 pg/ml (range 1-18) and 17 pg/ml (range 3.5-39), respectively. Following stimulation with PHA, the median IL-2 production by IEL and LPL cultured alone were respectively significantly increased to 2.5 IU/ml (range 1-26) and 9 IU/ml, the median IFN- γ production was 27 IU/ml (range 1-105) and 26.5 IU/ml (range 2-242), the median IL-10 production was 3 IU/ml (range 1-10) and 4 IU/ml (range 1-100). After 3 days of co-culture of IEL and LPL cells cytokine production was further increased to 7.5 IU/ml (range 2-340) for IL-10, 39.5 IU (range 8-450) for IL-2, and 104 IU/ml (range 18-500) for IFN- γ (Hoang 1992b, Sachdev 1993, Hoang 1997).

2.6 Release studies

Dose/response studies were performed in incubation media supplemented with known secretagogues, i.e. bombesin (10^{-9} to 10^{-5} mole/L) and phenylalanine (2-50 mmole/L). Cytokines tested were at the following concentrations: IL-1 (1,5,10,50 ng/ml), IL-2 (10 IU/ml), IL-4 (100 ng/ml), IL-6 (10, 1000 IU/ml), IL-10 (0.2, 20 ng/ml), TNF- α (70000 IU/ml), and Rantes (100 ng/ml). IEL or LPL supernatant studies were performed at a concentration of 10-50% (100 μ l added to 900 μ l of culture media). In those release studies the control media were supplemented with PHA when needed.

2.7 CCK extraction and RIA

Extraction and concentration of CCK from the culture supernatants and biopsies were immediately performed by adsorption on Sep-Pak C18 cartridges previously washed with 10 ml of acetonitrile, 10 ml of water and 10 ml of 0.1% TFA (Playford 1993). CCK was eluted with 3 ml of acetonitrile in 0.1% TFA (1:1, vol/vol) into a polypropylene vial. Eluates were lyophilised and stored at -20 °C for 1-4 weeks before performing radioimmunoassay. CCK concentrations were measured with a sensitive and specific RIA using Dino-7 antiserum as previously described (Jordinson 1996). Dino-7 antiserum was used at a final titre of 1:10⁶ with ¹²⁵I-Bolton-Hunter-labeled CCK-8 as a tracer and CCK-8 as a standard. It had similar affinities for all forms of sulphated CCK (CCK-8, CCK-33, CCK-39, CCK-58). Affinity was 3000 fold lower for sulphated and non-sulphated gastrin-17. The intra- and inter-assay variability of the assay was 6.2 and 12.1%, respectively. The detection limit of the assay was 0.2 pmol/L.

2.8 Statistics

Data were analysed on a logarithmic scale and reported as geometric means and 95% CI. The results for the CCK release were expressed as the percentage of total cells or biopsy specimen content. Groups were compared using the Student's t test or one-way

ANOVA with F-tests and post-hoc tests were performed when significant, using the Bonferroni method. A P value of less than 0.05 was considered as statistically significant.

3-Results

3.1 STC-1 cells

3.1.a Basal conditions

Basal secretion of CCK in the supernatant was 42.5 [CI 27-59] fmoles/ml/60 min. Mean CCK content of cells (10⁶ cells/well) was 1.4 [0.9-2.5] pmoles/10⁶ cells. Basal secretion ratio between CCK in the supernatant and in the cells was 3.5% [0.9-4.9].

3.1.b Releasing studies

Bombesin and phenylalanine significantly stimulated CCK release in a dose response manner (**figure 9**). IEL, PHA stimulated IEL, and LPL non-stimulated supernatants did not affect CCK release (3.3% [0.5-7.5]; 3.2 [0.8-5.6]; 3.1 [1.3-3.9] respectively). PHA stimulated LPL supernatant significantly decreased

Figure 9

CCK release in STC-1 cells (top panel) and short term organ cultures of human duodenal biopsies (bottom panel) incubated with increasing concentrations of bombesin (B₉= 10⁻⁹ to B₅= 10⁻⁵ M) and phenylalanine (PHE 2, 25 and 50 mM)

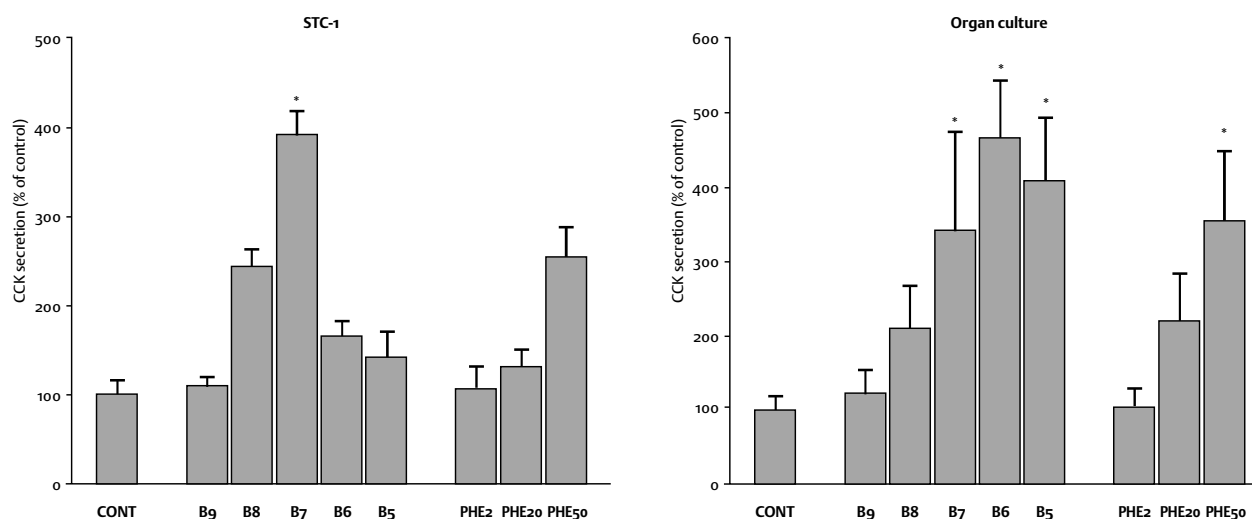
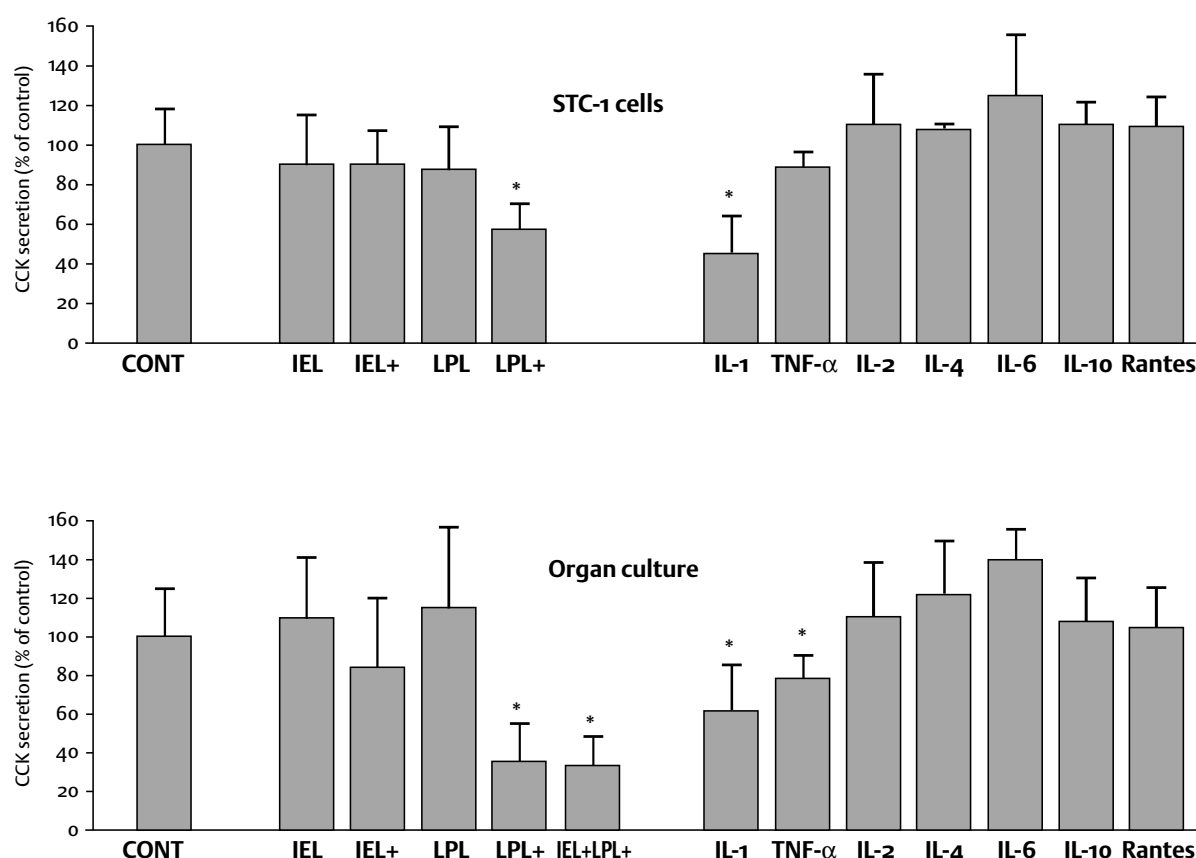


Figure 10

CCK release in STC-1 cells or organ cultures of human duodenal biopsies incubated with IEL or LPL supernatants and various cytokines. Cytokines were tested at the following concentrations: IL-1 (50 ng/ml), IL-2 (10 IU/ml), IL-4 (100 ng/ml), IL-6 (10 IU/ml), IL-10 (20 ng/ml), TNF-alpha (70000 IU/ml), and Rantes (100 ng/ml). IEL or LPL supernatant studies were performed at a concentration of 10-50% (100 μ l added to 900 μ l of culture media). * = $p < 0.05$.



CCK release to 1.9% [0.7-2.9]. The effect of various cytokines was dependent on the type and concentration tested. A significant inhibitory effect on CCK release was only observed with IL-1 at a concentration of 10 ng/ml (1.7% [0.5-4.6]), $p=0.04$). No significant effect was observed with TNF- α , IL2, IL-4, IL-10, IL-6 and Rantes (figure 10).

3.2 Organ culture

3.2.a Viability of organ culture

Histological appearance of the duodenal specimens was initially normal by light microscopy and showed a good preservation of villous architecture and epithelial cells during the 3 hours of culture. The villosity/crypt ratio was unchanged and the brush bor-

der was still visible. LDH ratio (supernatant/biopsy) was 4% at 2 hours incubation and 6.6% at 3 hours, which was considered as compatible with normal apoptosis. Viability of cells in organ culture was further confirmed by trypan blue dye exclusion and was greater than 94% in all cases.

3.2.b Basal conditions

Mean weight and protein content of duodenal specimens ($n=100$) were 6.7 mg [CI 5.1-7.8] and 134.1 μ g [119.9-148.4], respectively. CCK concentrations in the supernatant and the organ explants in non-stimulated conditions were 12.8 fmoles/ml [11.4-14.1] and 752.9 fmoles/biopsy [535.3-970.5], respectively. Basal release of CCK in control experiments was 0.10 fmoles CCK/ μ g protein [0.08-0.12] and 3.6% CCK expressed as a ratio between supernatant/explant [0.6-6.6].

3.2.c Releasing studies

Bombesin and phenylalanine significantly stimulated CCK release in a dose response manner (**figure 9**). Non-stimulated IEL, PHA-stimulated IEL, and non-stimulated LPL supernatants did not affect CCK release (3.9% [0.5-7.5]; 3.3 [0.8-5.6]; 3.8 [1.3-5.9], respectively). PHA stimulated LPL supernatant and supernatants from PHA stimulated co-culture of IEL and LPL significantly decreased CCK release to 1.5% [0.5-2.3], and 1.4 [0.8-2.1], respectively (**Figure 10**). The effect of various cytokines was dependent on the type and concentration tested. Inhibitory effect on CCK release was only observed with IL-1 at a concentration of 10 ng/ml (decrease to 1.6% [0.9-3.3], $p=0.04$) and TNF- α at 70000 IU/ml (decrease to 2.6% [1.0-4.9], $p=0.04$). No significant effect was observed with IL-2, IL-4, IL-10, IL-6 and Rantes (**Figure 10**).

4-Discussion

The role of the intraepithelial lymphocytic and the lamina propria infiltrates on CCK release has been studied in two models, a CCK releasing cell line STC-1 and short-term cultured normal duodenal specimens. In both studies, a significant inhibitory role on CCK release of the LPL supernatants could be observed. This effect was present only when supernatants were obtained from PHA-stimulated cultured lymphocytes. No inhibitory or stimulatory effect could be seen with IEL supernatant stimulated or non-stimulated with PHA. Supernatants obtained by co-culture of PHA-stimulated LPL and IEL had also a significant inhibitory effect.

Although there is clear evidence of a communication between the immune and neuroendocrine systems, very few direct interactions have been studied in the digestive area (Wassen 1996, Beales 1997, Prinz 1997, Beales 1998). Gastritis due to *Helicobacter pylori* infection is an example where an increased release of mucosal cytokines was clearly demonstrated (Weigert 1996, Beales 1997). In these patients, plasma gastrin levels are higher and cause an increased acid release linked to the occurrence of duodenal ulcers. IL-1 β and TNF- α were shown to stimulate gastrin secretion by receptors located on the antral G cells themselves (Weigert 1996). IL-8, although present in higher levels in the mucosa infected with *H. pylori*, had no effect on gastrin and somatostatin release (Beales 1997).

Elevated cytokine production can also be observed in other gastrointestinal diseases. Celiac disease is an interesting model of possible relationship between the mucosal inflammation and hormonal release.

Celiac disease is a gluten-induced enteropathy characterised by the presence of gliadin specific CD4+ T cells in the lamina propria and by a prominent intraepithelial T-cell infiltration (Jabri 2000). This infiltration is composed of both Th1 (IFN- γ , IL-2) and Th2 (IL-4, IL-10) cytokines with an expression associated with disease activity (Lahat 1999). There is a growing evidence to support that the T-cell mediated immune response to gluten causes the morphological modification observed at the various stages of the disease (Marsh 1992). As a result also, soluble mediators including cytokines are secreted, which may enhance the inflammatory response. Some of the mucosal changes could occur via the local production of cytokines. Particularly, the role of IEL that are observed at the early stages of the disease or in some celiac patients under gluten-free diet should be better defined (Deprez 2002b).

Celiac disease is also associated with a defective release of CCK that can participate to the malabsorption syndrome observed in these patients through deficient gallbladder contraction and pancreatic secretion. We observed in previous studies that a persistent defective CCK release was observed in those patients with a persistent IEL infiltrate. Our data does not provide an explanation for this phenomenon since an inhibitory effect was only seen with LPL or IEL-LPL co-cultured supernatants. Absence of visible effect could be due to the low production of cytokine by IEL even when they are stimulated by PHA, to a low sensitivity of our short-term assay to detect an inhibition of CCK release compared to a stimulation of release or to different phenotypes between cultured lymphocytes originating from the colon versus those observed in the small intestine. It should be remembered however that colonic lymphocytes react to gliadin antigens in the same manner than duodenal lymphocytes.

Of the putative cytokines involved in the interaction between immune and neuroendocrine systems, only IL-1 and TNF- α played an inhibitory action in our experimental model. Increased presence of these cytokines has been described in celiac disease and could therefore play a role in the defective CCK release observed in these patients. Inhibitory actions of these cytokines has been demonstrated on gastrin and somatostatin release. On the other hand, IL-1 was also shown to stimulate CRF, ACTH, GHRH and SRIH in other experimental models (Tsagarakis 1989, Honegger 1991, Gwosdow 1994, O'Connell 1994, Gonzalez-Hernandez 1995, Deprez 2002b). Elevated levels of other cytokines have been shown in celiac disease (TNF- α , IL-2, IL-6, IL-4, IL-10) (Lahat 1999). We were particularly interested in those cytokines with a putative suppressive action (IL-4, IL-10). We however did not detect any inhibitory effect on CCK release. Finally we tested IL-2 and IL-6 that were shown to be stimulants for GHRH and CRF release, respectively (Honegger 1991, Karanth 1993). Some

stimulatory effect was shown for IL-6 in organ explants.

In conclusion, the interaction of the neuroendocrine and immune systems has been further illustrated by the inhibition of CCK release in SCTC-1 cell line and in short-term culture of normal duodenal explants with supernatants from stimulated cultured lamina propria lymphocytes. These data could partly explain the impaired CCK

release observed in celiac patients. When individual cytokines were tested, some did inhibit release of CCK whereas most of them did not influence significantly hormonal release. Modulation of CCK release in the gut is therefore a complex process implying co-regulation of nutrients, releasing peptides, neurotransmitters and soluble factors including cytokines.

SYNTHESIS AND CONCLUSIONS

The aim of this work was to study the defective CCK release in adult patients with celiac disease. The decreased CCK secretion was first shown to be caused by a reduction in the number of CCK cells and their hormonal content in the presence of mucosal atrophy but other mechanisms have been postulated: a lack of stimulation of the mucosal CCK cells due to impaired intra-duodenal hydrolysis of nutrients, a functional abnormality of the CCK-cell, an impaired interaction of the CCK-cell with other cells (endocrine, epithelial, immune or nervous cells), the absence of a stimulating factor (e.g. luminal cholecystokinin releasing factor) or the presence of inhibitory factors.

Celiac disease is characterized by malabsorption resulting from inflammatory injury to the intestinal mucosa after the ingestion of wheat gluten or related rye and barley proteins. The lesions observed in the mucosa consist in a spectrum of changes classed as preinfiltrative, infiltrative, hyperplastic, destructive and hypoplastic types. The studies concerning CCK in celiac disease have evaluated patients with a typical flat lesion (destructive or hypoplastic type). We however know that even in histological changes of lesser degrees functional abnormalities of the gut have been reported. These “early” changes are now more often seen at the time of celiac disease diagnosis and are frequently observed in celiac patients who follow a strict gluten free diet. We moreover observed that some of our adult celiac patients, while treated with a strict gluten-free diet, still present with abdominal complaints and diarrhea that are related to steatorrhea and pancreatic/biliary insufficiency. These patients may indeed benefit from pancreatic enzyme supplementation.

We therefore studied CCK release in various types of celiac mucosal lesions with measurements of basal and meal stimulated plasma CCK levels. We studied the relationship between the distinctive mucosal patterns and the mucosal CCK and CCK mRNA content. We compared the effects of a polymeric or a semi-elemental liquid

test meal on CCK release in controls and in the different groups of celiac patients. Finally, to evaluate the immuno-endocrine interactions suggested by our first results, we examined the role of the mucosal inflammatory infiltrate on CCK release from a murine neuroendocrine tumour cell line STC-1 and from short-term

organ cultures of duodenal biopsy specimens.

We have shown that CCK release is not only impaired in untreated celiac patients with a destructive/hypoplastic type duodenal mucosa but also in patients whose mucosa solely displayed a significant intraepithelial lymphocytic (IEL) infiltrate. Only treated celiac patients without hypoplastic or destructive mucosa and without any increase in IEL infiltrate have a basal and postprandial CCK release similar to that of the control group.

When using immunocytochemistry and semi-quantitative PCR we have demonstrated that the defective release of CCK observed in patients with coeliac disease is not related to a decrease in the number of CCK cells present in the proximal part of the small intestine but rather to a decrease in CCK synthesis related to a decrease in mRNA content, even in patients with an infiltrative type of celiac disease. These data suggest that the inflammatory infiltrate in the mucosa of celiac patients affects expression of the CCK gene.

The next part of our work indicates that ingestion of a pre-digested meal does not correct the defective CCK release in patients with a destructive or an infiltrative type of celiac mucosal lesion. Moreover intraduodenal food hydrolysis should be considered normal in celiac patients with mucosal lesions limited to an intraepithelial lymphocytic infiltrate. The decreased levels of plasma CCK in this group of patients, given a pre-digested meal, strongly support that the

defective CCK release could be related to suppressive factors released by the inflammatory infiltrate, rather to a lack of stimulation due to impaired intraduodenal nutrient hydrolysis.

We therefore studied the effects of supernatants obtained from IEL and lamina propria lymphocyte cultures and various cytokines produced by mucosal lymphocytes on short-term organ cultures of duodenal biopsy specimens obtained from normal controls and on the CCK secreting murine neuroendocrine tumour cell line STC-1. We demonstrated a significant inhibitory effect of supernatants obtained from phytohaemagglutinin A-stimulated cultured lamina propria lymphocytes and from phytohaemagglutinin A-stimulated co-cultured lamina propria and intraepithelial lymphocytes, and of interleukin-1 and tumour necrosis factor- α on CCK release.

All together these data show that the defective CCK release is present at the initial infiltrative type of mucosal lesion seen in celiac disease and that this defective release may be related to inflammation and suppressive factors associated with the inflammatory cells. We conclude that modulation of CCK release in the gut should be considered as a complex process implying co-regulation of nutrients, releasing peptides, neurotransmitters and soluble factors including cytokines. We provide evidence of a relationship between intestinal endocrine cells and inflammation.

PERSPECTIVES

The gut mucosa should be regarded as a complex sensory organ with three types of detectors (neurons, endocrine cells, and immune cells) mixed with epithelial cells. The gastrointestinal tract has an integrated response to changes in its luminal content.

When this response is altered (i.e. in celiac disease) the consequences can be severe and debilitating. It is thus essential to obtain a full understanding on how the gastrointestinal tract reacts to the information and how epithelial cells, neural, hormonal and immune signals interact (Buchan 1999, Furness 1999).

The classical views on dysfunction of gut endocrine cells in various diseases have to be challenged when new experimental data is produced.

In the same way we challenged the classical explanation for hypergastrinemia in different inflammatory or autoimmune diseases (Deprez 1991a, Deprez 1991b, Deprez 1993), our present data raise several questions on the intracellular mechanisms of CCK cells dysfunction in celiac disease, on the precise interaction between CCK cells and intraepithelial or lamina propria lymphocytes and on the respective roles of the various inflammatory mediators on the digestive endocrine system.

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