EFFECTS OF LECTINS IN LEGUME SEEDS ON THE STRUCTURE AND FUNCTION OF THE SMALL INTESTINAL MUCOSA

In vivo and *in vitro* studies

MARIA J.L. KIK

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Drukwerkverzorging ADDIX
Wijk bij Duurstede

10

Voor de erkende niet-veterinaire beoefenaren van pathologie bij laboratorium dieren is "dierproefpatholoog" een betere benaming dan proefdierpatholoog".

11

Het geregistreerd zijn als veterinair patholoog verdient ook een daadwerkelijke universitaire erkenning.

12

Het welslagen van een onderwijsvernieuwing is primair afhankelijk van een juiste onderwijscultuur.

13

Het verrichten van wetenschappelijk onderzoek, in het bijzonder patiëntgebonden wetenschappelijk onderzoek, gaat in het algemeen gepaard met een kritische instelling ten opzichte van schijnbaar gevestigde inzichten.

14

Het scheiden van onderwijs en onderzoek in de diergeneeskunde is een idee dat alleen kan opkomen bij mensen die niet zelf op beide terreinen werkzaam zijn geweest.

15

Onzekerheidskunde is een grondbeginsel van de diergeneeskunde. Onderzoekers zijn daarvoor de meest geschikte leermeesters.

16

Toekomstige onderzoekers onder de studenten gaan verloren als docenten niet tevens onderzoeker zijn.

Stellingen behorende bij het proefschrift "Effects of lectins in legume seeds on the structure and function of the small intestinal mucosa. *In vivo* and *in vitro* studies" van Maria JL Kik. 24 januari 1991

S. 16.654

STELLINGEN

1

Wanneer Suis domesticus de kans had gekregen zou het de hond hebben vervangen als de beste vriend van de mens in plaats van zijn beste bron van eiwit te zijn.

2

Wanneer dieren in handen van de veterinair patholoog vallen hebben ze ondanks dat ze zwijgzaam zijn toch veel te vertellen (Vos, 1990).

3

Gifslangen zijn niet uitgerust met een gif apparaat omdat ze ooit homo sapiens zouden ontmoeten (Phelps).

4

Conventionele biggen zijn vergeleken met S.P.F. biggen minder geschikt in de studie naar pathologische effecten van antinutritionele factoren op de structuur en functie van het dunne-darmslijmvlies.

5

Bij de bestudering van de effecten van voedingsmiddelen op een dier dient men dusdanige methoden te hanteren dat lijden van het dier tot een minimum wordt beperkt.

6

De voorkeur voor het contracteren van vrouwen aan de Faculteit Diergeneeskunde der R.U.U. dient zich uit te breiden tot hoogleraarsfuncties.

7

Misschien denken SPF-varkens over "varkens de la rue" net zo laatdunkend als huishonden over straathonden (promotiegroep ANF).

8

In tegenstelling tot wat de naam doet vermoeden, worden "varkens de la rue" gehuisvest en wel onder gecontroleerde omstandigheden.

9

Een op rauwe soja-produkten gebaseerd vermageringsdieet is medisch niet verantwoord.

EFFECTS OF LECTINS IN LEGUME SEEDS ON THE STRUCTURE AND FUNCTION OF THE SMALL INTESTINAL MUCOSA

In vivo and *in vitro* studies

Effecten van lectinen uit zaden van vlinderbloemigen op de structuur en functie van het slijmvlies van de dunne darm

In vivo en *in vitro* studies

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Rijksuniversiteit van Utrecht op gezag van de Rector Magnificus, Prof. Dr. J.A. van Ginkel, ingevolge het besluit van het College der Decanen in het openbaar te verdedigen op donderdag 24 januari 1991 des namiddags te 14.30 uur

DOOR

MARIA JACOBA LEONARDA KIK

Geboren op 24 juni 1956 te 's-Gravenhage

Promotor : Prof. Dr. J.M.V.M. Mouwen
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TNO Institute for Animal Nutrition and Physiology (ILOB), Wageningen, The Netherlands.

Aan mijn moeder
Ter nagedachtenis aan
mijn vader

The research conducted in this study was financed by TNO Nutrition and Food Research, Zeist, the Netherlands and the Commodity Board for Feed stuff and financially supported by the "Commissie bevordering diergeneeskundig en vergelijkend ziektekundig onderzoek". The research has been executed in cooperation with the TNO Institute for Cereals, Flour and Bread (IGMB), the TNO Institute for Animal Nutrition and Physiology (ILOB) and the Department of Animal Nutrition, Agricultural University, Wageningen, The Netherlands. Resulting in two other theses:
Antinutritional effects of legume seeds in piglets, rats and chickens, by J. Huisman
Effects of processing on bean (*Phaseolus vulgaris L.*) protein quality, by A.F.B. van der Poel.

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CHAPTER I

GENERAL INTRODUCTION

INTRODUCTION

Legume seeds, e.g. beans and peas, play an important role in the nutrition of human beings all over the world[6]. Over the past years more seeds of legumes (e.g., *Phaseolus vulgaris, Pisum sativum, Vicia faba, Glycine max*) are also included in diets for monogastric farm animals as source of protein. However, the presence of a number of antinutritional factors (ANF) such as protease-inhibitors, tannins and lectins in the raw beans and peas is a disadvantage to their consumption. These seeds, when fed to human beings or animals can be highly toxic, unless subjected to some form of heat treatment, although heat processing is not always very effective[10,13].

It is generally assumed that the lectins among the ANF in the *P. vulgaris* beans account for the major part of the pathological changes evoked by feeding of untreated legume seeds[1]. These and other lectins, e.g., *Concanavalin A*, wheat germ, and soybean agglutinins, which can reach the intestinal tract in an active form, induce severe pathological changes of the small intestinal mucosa of laboratory animals. Exposure of the small intestine to lectins leads to villus atrophy and elongation of crypts, alteration of microvilli, and diminished activity of brush border enzymes[2,7,9,11]. In pigs, however, after feeding of *P. vulgaris* beans morphologically only changes of the brush border were detected[5]. Short-time feeding experiments with the addition of *P. vulgaris* or *P. sativum* to the diets revealed negative effects on protein utilization, life weight gain, and feed conversion in pigs[3,4,8,12].

However, the pathogenesis of lectin-induced changes of the small intestinal mucosa is not yet fully understood.

The purpose of the studies conducted in this thesis is to develop *in vivo* as well as *in vitro* models to gain insight into the nature and pathogenesis of the effects of plant lectins on the mucosa of the small intestine in pigs.

First the literature concerning the interaction of plant lectins with and their effect on the small intestinal epithelium is reviewed (**Chapter II**).

The effects of crude *P. vulgaris* (red kidney) beans on the structure and function of the small intestinal mucosa in piglets were studied *in vivo* after dietary administration (**Chapter III**).

Further, pathogenetic studies were carried out *in vitro*. The binding and effects of *P. vulgaris* isolectins E4 and L4 were studied in cultures of human colon carcinoma Caco-2 cells (**Chapter IV**). For better extrapolation possibilities to *in vivo* situations, further studies on binding and effects of purified isolectins were conducted in organ cultures of small intestinal mucosa of pigs (**Chapter V**).

Finally, to study the effects of purified lectins *in vivo*, a small intestinal mucosal biopsy technique in cannulated piglets (**Chapter VI**) and a self

emptying blind pouch model were developed (**Chapters VII and VIII**).

The *in vitro* study in the Caco-2 model comprises the effects of purified isolectins on the incorporation of radioactive precursors for synthesis of DNA, RNA, and (glyco)protein. The morphological investigations comprise stereomicroscopical, histomorphometrical, immunocytochemical, scanning electon microscopical, transmission electronmicroscopical, and immuno-electron microscopical examinations. The activities of the brush border enzymes sucrase-isomaltase and aminopeptidase were assessed as criteria for typical functioning of the brush border membrane of the small intestine[9].

The piglets used in the study of the effects of raw *P. vulgaris* beans *in vivo* were conventionally bred and kept animals. To diminish unknown effects of environmental influences, the piglets used in the development of the *in vivo* and *in vitro* models were specific pathogen free bred and housed behind barriers.

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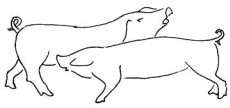
CHAPTER II

THE INTERACTION BETWEEN PLANT LECTINS AND THE SMALL INTESTINAL EPITHELIUM: A PRIMARY CAUSE FOR INTESTINAL DISTURBANCE



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Summary

The literature concerning the effects of plant lectins on the small intestinal epithelium is reviewed. It appears that after oral intake, intact plant lectins can reach the small intestinal lumen. Their binding to the mucosal surface evokes an increased synthesis of glycoproteins and a degeneration of the intestinal epithelium. The epithelial alterations may result in hyperregenerative villus atrophy and endogenous nitrogen loss. These changes ultimately can lead to less efficient feed conversion, diminished growth, scouring, wasting and death. The possible significance of plant lectins in digestive disturbances in farm animals is suggested.

Introduction.

Over the past years, more seeds of legumes have been added, for a number of reasons, to animal diets as a source of protein. Since the protein content of cereals is usually too low for optimal growth of young farm animals, supplementation with sources of a higher protein content is necessary. Furthermore, seeds of legumes supposedly replace the more expensive protein sources such as animal protein[32,34,45]. A reason for the relative cheapness of legume seeds within the Common Market is that these seeds are highly subsidized[54]. However, the presence of antinutritional factors (ANF) in almost all the legume seeds is a disadvantage of legume consumption. The ANF, such as protease inhibitors, tannins and lectins, are the cause of less efficient utilization of the proteins[33,45]. The consequences of ANF for production animals are less efficient feed conversion, diminished growth, intermittant scouring, wasting and sometimes death, perhaps resulting from damage to the small intestinal mucosa[3,21,28,45].

The purpose of this literature study is to gain an insight into the nature and pathogenesis of the effects of plant lectins on the epithelium of the small intestine.

Lectins: nomenclature, occurrence, structure and function.

In 1888, a description of what we now know to be a lectin had already been given by Stillmark[51]. In 1954, Boyd and Shapleigh[4] proposed the term lectin (from the latin "legere": to choose or to pick out) for a group of plant extracts with the capacity of agglutinating erythrocytes. More recently, lectins have been refered to as plant seed derived carbohydrate binding proteins, which can be classified according to their carbohydrate binding specificity[19]. Because of their haemagglutinating ability, the lectins are also called phytohaemagglutinins (PHA), but this term is in fact an accepted designation for the lectins of the red kidney bean (*P vulgaris*) only[19]. Liener[33] proposed the term lectin for all proteins with a specific affinity for carbohydrates, and depending on their origin the terms phyto-, zoo- or mycolectin may be used. Furthermore, lectins which do not agglutinate cells, but have distinct mitogenic or toxic features, could be called mitogenic or toxic lectins. However, according to Goldstein et al[20], the toxic lectins are not lectins. He refers to lectins as proteins or glycoproteins of non-immune origin which are able to agglutinate cells or to precipitate glycoconjugates. Franz and Ziska[18] consider glycoproteins or proteins with one or more sugar specific combining sites which are not enzymes or antibodies, to be lectins.

Lectins occur widely in nature, and are encountered not only in plants but also in viruses, fungi, bacteria, invertebrates and vertebrates[6,19,21]. In legumes,

the lectins are especially found in the seeds, which may contain 1-3% of lectins[30].

The molecular weight of lectins varies from 25,000 to over 400,000, whereas the sugar content may range from 0-20%. Thus the lectins form a heterogenous group of mostly glycoproteins[30].

In general, a lectin molecule consists of one or more units, depending on temperature and pH. A decrease in the number of units in one complex signifies a diminution of its agglutinating ability[25,30]. The structure of the lectins has been most intensively studied in Concanavalin A (ConA) derived from *Canavalia ensiformis*, jack bean[6,25,30]. Its polypeptide chain consists of 273 amino acids[49]. A dimeric or tetrameric form of ConA exists, in which each unit is identical and has a molecular weight of approximately 26,000[25,30]. A solution of ConA contains a mixture of dimeric- and tetrameric forms[6,25,30],. The binding of the units to each other is an electro-covalent one[30]. Every single unit of the ConA molecule has, as in most lectins, a carbohydrate binding site. Soy bean agglutinin (SBA) is an exception, in that the number of its saccharide binding sites is half the number of its subunits[56]. It has a molecular weight of 120,000 and forms a tetramer comprised of 4 identical units. Ricine (RCA-1: *Ricinus communis* agglutinin 1) from *Ricinus communis* (castor bean) is known to have a dimeric structure composed of two units (an A- and a B-polypeptide chain) bound to each other with a covalent disulphide bridge[30,44]. The structure of a steadily growing number of lectins has been elucidated.

The biological function of lectins is not well known. In plants they may play a role as storage proteins, and are possibly of significance in the embryological development and germination of seeds[24,41], the transportation and storage of sugars, protection of plants against viruses, bacteria, fungi and insects, and the symbiotic interaction between leguminous roots and nitrogen fixing bacteria[19,21,30]. In bacteria, the lectins may function in the adherence to host cells. In animals the endogeneous lectins (e.g. the membrane lectin of liver cells[38], and the lactose binding lectins in adult chicken intestine[52]) are involved in endocytosis and intracellular translocation of glycoproteins, regulation of cell migration and adhesion, and as mediators of phagocytosis[50]. Torres-Pinedo[52] states that endogeneous lectins may play an important role in the localization and stabilization of cell surface glycoproteins. Feizi and Childs[17] suggest that endogeneous lectins can modulate the intracellular response to the presence of epidermal growth factor (EGF).

The binding of plant lectins to small intestinal epithelium.

Lectins are able to bind to carbohydrate chains of glycoproteins and glycolipids[30]. Both the membrane bound glycoconjugates of the glycocalyx[11] and the free glycoconjugates in the mucus[39] are considered targets for this

binding. The glycocalyx on the surface of the intestinal microvilli is composed of the oligosaccharide chains of membrane bound glycoconjugates, which are produced by the epithelium. The mucus layer on the mucosal surface consists of water and glycoconjugates, which are produced by the goblet cells. Specific moieties or patterns of moieties in a carbohydrate chain may function as receptors for all kinds of substances, including lectins[11,39].

Enteroblasts from the bottom of the crypts of Lieberkühn differentiate into mature enterocytes towards the top of the villi. During this maturation the carbohydrate chains in the membrane bound glycoconjugates also change[15,16,56], a conclusion reached on the basis of different lectin binding between the epithelial cells of the crypts and those of the villi. Köttgen *et al*[31] state that the enzyme glucosamine-N-acetyl-transferase-1 plays an important role in the process of turnover of the membrane bound glycoproteins.

Since lectins react with any cell surface glycosyl moiety that is exposed and complementary to them, the term "lectin receptors" should not be used[19]. The term receptor should be reserved for those membrane structures that bind external molecules in a highly specific way, thus transmitting signals from the environment to the interior of the cell. However, Etzler[14] does use the term receptor, indicating sites on the surface of the epithelial cell where different lectins can bind. Kauss[25] uses both terms. So in this respect the terminology used in the literature is contradictory.

Although the specificity of individual lectins for carbohydrates is mostly restricted to certain monosaccharides, some lectins are less specific and bind to several structurally related monosaccharides[20]. However, it is of no importance with regard to the binding itself whether monosaccharides occur as free units or are part of oligosaccharides or more complex carbohydrates, such as the membrane bound glycoconjugates[6]. The binding of a lectin to a glycoconjugate depends as a rule on factors such as lectin concentration, pH and temperature, according to a saturation pattern. On the other hand, the binding also depends on the anomeric form in which the monosaccharides are presented, the spatial arrangement, and the distance of the monomeric units. The affinity of lectins for the complex saccharide sequence of the membrane bound glycoconjugates is higher than for monosaccharides[19]. This is attributed to the fact that most lectins possess several sugar binding sites, suggesting that a lectin can bind to more than one site of the carbohydrate chain of the glycoconjugates and thus form a more stable complex[19]. Köttgen[30] states that lectins can also bind to internal sugars of a carbohydrate chain. The protein part of the glycoprotein plays a role in the interaction with a lectin[19,25].
According to the 'fluid-mosaic' membrane model, the membrane-bound glycoconjugates can move laterally within the plane of the membrane[5,6]. This movement can be restricted by structures such as extracellular mucus, tight junctions, and elements of the cytoskeleton[11,43]. Sometimes lectin 'receptors' can be in contact with the cytoskeleton[6].

Etzler[14] was able to demonstrate at least three different lectin receptors on the surface of epithelial cells of the rat small intestine, by means of lectins labelled with fluorescein isothiocyanate. Those lectins emanated from *Lotus tetragonolobus* (asparagus pea), *Ricinus communis* (castor bean), *Dolichos biflorus* and wheat germ. These findings suggest that the surface of the microvilli is rich in alpha-D-fucosyl (*Lotus tetragonolobus* lectin-binding), non-reducing beta-D-galactosyl (RCA-1 binding), beta-N-acetyl-glucosaminyl (wheat germ agglutinin (WGA) binding) and N-acetyl-D-galactosamine (*Dolichos biflorus* lectin-binding) residues in a terminal position, which can bind the above mentioned lectins[14,15,56]. Exclusively *Dolichos biflorus* (horse gram) lectin attaches also to the contents of goblet cells[14]. The binding of other lectins with the contents of the goblet cells depends on the localization of these cells in the small intestine: the more distal in the small intestine the more binding to goblet cells occurs[14]. According to Etzler and Branstrator[15], the difference in lectin binding between the various goblet cells can be ascribed to the phase of mucin synthesis or the synthesis of a different mucin.

By an indirect immunofluorescence method, King *et al.*[27] were able to demonstrate binding sites for PHA in the epithelium of rat small intestine. The major part of the lectin binding sites were detected in the brush border regions of the enterocytes of the proximal small intestine. Lectin binding sites could also be detected in the mucus layer on the mucosal surface. In addition, small lectin particles were found in the Golgi apparatus of the enterocytes at the top of the villi[27].

Recently, Hendriks *et al.*[23] developed a method to determine quantitatively the amount of soybean lectin binding sites in isolated small intestinal brush border membranes of calves.

Cortisone and thyroxine appear to influence lectin binding in the small intestine. When administered to eight day old rats, cortisone caused diminished binding of WGA and augmented binding of 125I-*Ulex europaeus* agglutinin. The administration of thyroxine resulted in augmentation of the binding of 125I-*Ulex europaeus* agglutinin[38].

Pathogenesis, functional consequences, and pathological effects of the binding of plant lectins to the small intestinal epithelium.

Only those lectins that are not completely degraded in the digestive tract may be toxic to the intestinal epithelium[42]. Greer[21] proved that PHA could withstand intestinal proteolysis when fed orally, by demonstrating the presence of PHA in the faeces of rats. Hara *et al.*[22] proved that the kintoki bean lectin was not inactivated in the stomach of mice, but reached the small intestine in an active form, where it could bind to the epithelium. A large amount passed through as free lectin, which retained its agglutinating capacity. Nakata and Kimura[42] have demonstrated ConA in the digestive tract

and faeces of rats after oral intake.

The intact lectins can bind to the carbohydrate chains of glycoconjugates[30], present in the mucus[39] and in the glycocalyx[11]. The consequences of this binding may generally consist of a precipitation of glycoconjugates of the mucus and a damaging effect on the intestinal epithelium[21,30]. The precipitation of the glycoconjugates of the mucus may have consequences for the integrity of the outermost mucosal barrier[39]. The binding of lectins to the glycocalyx can influence the activity of various membrane bound enzymes[13,42]. The presence of 0.3 or 0.5% ConA in a diet completely suppresses the adaptive changes in the activity of sucrase, alkaline phosphatase and leucine aminopeptidase in rats *in vivo*. This contrasts with the adaptive increase of the activity of these enzymes when a diet with a high sucrose concentration is offered. These changes in enzyme activity occurred with lectin concentrations which were able to precipitate enzymes *in vitro*. Inhibitory sugars could prevent the disturbance of the enzyme activity, which suggests that the effect is due to the binding of lectins to specific carbohydrate structures[13,26]. Lorenz-Meyer *et al.*[35] have demonstrated similar effects in rats *in vivo*. Lorenzsonn and Olson[36] and King *et al.*[27] noticed injury of the cytoskeleton of enterocytes by WGA and ConA. SBA added to a human colon carcinoma cell line (CaCo-2), had an effect on the cytoskeleton of these cells, consisting of a conversion of F-actin into G-actin[9]. After oral intake of PHA, Greer[21] found an increase of mucus production. Rouanet *et al.*[48] noted a shortening of the microvilli and desquamation of the enterocytes after feeding diets containing PHA to rats. Lorenz-Meyer *et al.*[35] conclude that long term administration of WGA and ConA to rats leads to structural changes in the small intestinal mucosa. They observed a reduction in the length of the villi and an increase in the depth of the crypts. This hyperregenerative villus atrophy resembles the subtotal villus atrophy described in Gluten Sensitive Enteropathy (GSE) in man, the pathogenesis of which may be related to the lectin properties of gluten[2,8,30].

Little is known about the pathogenesis of the effects of lectins on the intestinal epithelium. Rossi *et al.*[47] state that purified kidney bean lectins play a direct role in the pathogenesis of microvillus abnormalities of the enterocytes in the proximal jejunum of rats. Lorenzsonn and Olson[36], were able to prevent damage to the small intestinal epithelium of rats, caused by WGA and ConA, by administering N-acetyl-D-glucosamine (WGA inhibition) and mannan (ConA inhibition). The conclusion was that the effects of WGA and ConA can be ascribed to their carbohydrate binding properties. Lectins may interfere with the proliferation and maturation of intestinal epithelial cells by inhibiting the binding of epidermal growth factor (EGF) to the EGF-receptor on the intestinal epithelium[17]. Besides, lectins bound to EGF/EGF-receptor complexes may form a macromolecular unit with other adjacent membrane bound glycoconjugates, thus modulating the expression of EGF[17].

RCA-1 is taken up by the cell through endocytosis after attachment of the B-chain to the membrane binding site. Intracellularly, the A-chain splits off from the complex and attaches to the ribosomes, where it manifests itself as an inhibitor of protein synthesis[30,44].

According to Wilson *et al.*[55], bacteria play an important role in the pathogenesis of the toxic effects of the red kidney bean lectins in rats. They observed a dramatic increase of the numbers of E. coli in the small intestine of rats fed diets with high concentrations of PHA. Rattray *et al.*[47] state that the toxicity of PHA is significantly reduced in germ free rats. A possible explanation for this phenomenon is that lectins might enhance the virulence of *Escherichia coli* by eliminating competitive bacteria. According to Greer[21] and Pusztai (45), lectins have an immunosuppressive effect, causing an increase in the growth of bacteria in the intestine. King *et al.*[28] suggest that *E. coli* bacteria could be able to bind to dietary lectins bound to the cell membrane and by doing so could increase internalisation of surface-bound enterotoxin.

In rats, the oral intake of PHA causes impaired growth, intestinal maldigestion and malabsorption[3,21]. The malabsorption may be caused by damage of the enterocytes and villus atrophy[28,35,36]. Banwell *et al.*[1] suggest that PHA may alter the absorption of nutrients by its effects on transport across the intestinal membrane. From studies with rats, Greer[21] has shown that PHA leads to a negative nitrogen balance. The elevated loss of nitrogen can be attributed to disturbance of normal digestion and absorption on the one hand and increased endogenous nitrogen loss on the other. The endogenous nitrogen loss is caused by shedding of damaged cells, increased production of mucus, and loss of plasma proteins to the intestinal lumen. Dobbins *et al.*[7] suggest that dietary lectins (PHA) may influence the regulation of intestinal fluid and electrolyte transport in rabbits. Soybean products have a clear effect on ligated small intestinal segments in swine, since an augmented net secretion of fluid was observed after injection of these products in the intestinal segments. The factor responsible for this effect was heat stable[40].

Up to 5 % of the oral intake of lectins can be found in the circulation[21]. This uptake of lectins could possibly happen through endocytosis by the enterocytes and the M-cells[12] or by means of a (para)cellular diffusion through the intestinal epithelium after injury of the cell membranes. The degree of absorption of macromolecules by the enterocytes also appears to be related to the concentration of macromolecules (e.g. lectins) in contact with the apical membrane of the epithelial cell[53]. Finally, repeated uptake of lectins may evoke food allergy[9].

Conclusions

From the literature it becomes evident that after oral intake, lectins can reach the small intestine, where they can bind to the polysaccharide chains of membrane-bound glycoconjugates at the level of the glycocalyx. This binding can result in degeneration of the villus epithelium and an increase in glycoprotein synthesis. The degenerative changes consist of a disruption of the microvilli, alteration of the cytoskeleton, and a change in activity of the brush border enzymes. Elevation of the glycoprotein production might lead to augmentation of the amount of mucus.

Although little is known about the pathogenesis of these changes, lectin induced dysbacteriosis also may play a role. Moreover, damage to the integrity of the mucus layer by lectins and a possible EGF modulation may be of importance. From studies on *Ricinus communis* agglutinin it is known that the RCA-1 component, after endocytosis, can inhibit the synthesis of proteins. Degeneration of the villus epithelium results in a hyperregenerative villus atrophy. Villus atrophy accounts for maldigestion and malabsorption, while increased glycoprotein synthesis can account for the elevated endogenous nitrogen loss. These changes ultimately can lead to less efficient feed conversion, diminished growth, scouring, wasting and death.
The deleterious effects of plant lectins, mostly described in laboratory animals, may also be of significance in farm animals.

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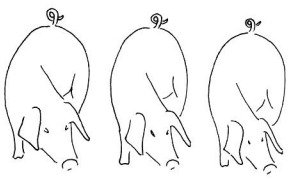
CHAPTER III

PATHOLOGIC CHANGES OF THE SMALL INTESTINAL MUCOSA OF PIGLETS AFTER FEEDING OF *PHASEOLUS VULGARIS* BEANS



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Abstract.

The jejunal mucosa of piglets fed diets containing *Phaseolus vulgaris* beans was characterized grossly as mucosal atrophy and microscopically as atrophy and blunting of the villus in association with elongation of crypts with cells with increased mitotic activity. These morphological findings were most severe in the proximal and middle parts of the jejunum. Compared to controls, goblet cells were significantly decreased in the villus but markedly increased in the crypt region. The activity of aminopeptidase and sucrase-isomaltase in the test animals was also significantly lower than in the controls. The findings in this study suggest that feeding with *P. vulgaris* beans reduces the digestive and absorptive capacity of the mucosa, resulting in weight loss and diarrhea of affected piglets.

Introduction

Seeds of legumes may be highly toxic to human beings and animals.[12,19] These seeds contain a number of antinutritional factors, like protease inhibitors, tannins, and lectins, which may account for a less efficient utilization of the plants' proteins[11,13]. Pusztai *et al.*[20,21] suggested that when legume seeds are fed orally, the lectins chiefly account for the morphological and functional changes in the epithelium of the small intestine in human beings and animals. In an active form, the lectins can reach the intestine[5,6,9], where they can bind to the carbohydrate chains of glycoconjugates, present in the mucus[17] and the glycocalyx.[3] This may interfere with the integrity of the mucus layer on the mucosal surface[17] and the activity of the brush border enzymes[4,18,23]. Greer[5] found an increased mucus production in rats after oral intake of *P. vulgaris* agglutinin (PHA). Other researchers have noted a shortening and disruption of the microvilli and desquamation of the enterocytes of the rat[9,23], while Lorenz-Meyer *et al.*[13] observed a reduction of the length of the villi and an increase in the depth of the crypts.

Over the past years, more seeds of legumes, containing anti-nutritional factors have been used in complete feeds for food producing animals (e.g., piglets) as a source of protein. Without proper heat treatment, the inclusion of *P. vulgaris* cultivars in diets for piglets leads to a less efficient feed conversion, diminished growth, intermittant scouring, wasting, and sometimes death. These symptoms suggest damage to the small intestinal mucosa, although the pathogenesis of such symptoms in piglets is largely unknown. The purpose of this study is to investigate morphologically and biochemically the jejunal mucosa of piglets after feeding diets containing *P. vulgaris* beans.

Material and Methods

Experimental Design

Twenty-five Great Yorkshire and Dutch landrace crossbred piglets were weaned at 2 weeks of age. They were then adapted to test cages for 2 weeks. The piglets were randomly assigned to two groups of seven animals each and one group of 11 animals and were introduced to the test or control diets for 3 days (Table 1).

The basic nutrient composition of the diets may be found in Table 2. The diets were formulated so that each contained comparable levels of digestible protein (15.5%) and metabolizable energy (16.3 MJ per kg). The *P. vulgaris* bean protein digestibility was calculated to be 0 and 60% for the test-diets I and II respectively. The animals were fed at a level of 2.2 times maintenance requirement for energy. Maintenance was assumed to be 0.42 MJ metabolizable energy per kg metabolic weight ($kg^{0.75}$). The testing period

Table 1. The composititon of the control diet and test diets of three groups of piglets and the percentage of *Phaseolus vulgaris* in each ration.

	Diet	Composition	Number of animals
1.	control	Semisynthetic diet. No *Phaseolus vulgaris* beans included.	7
2.	test I	Semisynthetic diet. 20% (w/w) untreated *Phaseolus vulgaris* beans added. The bean protein digestibility was assumed to be zero.	11
3.	test II	Semisynthetic diet. 20% (w/w) untreated *Phaseolus vulgaris* beans added. Digestibility of the bean protein was assumed to be 60%.	7

Table 2. Detailed analysis of the nutrient composition (g/kg) of the experimental ration.

	Control Diet	Test Diet I	Test Diet II
Crude protein	168	210	184
Digestible protein	155	155	155
Ash	60	64	64
Crude fat	41	74	60
Crude fiber	25	25	25
Calcium	96	96	96
Phosphorus	80	80	80
Digestible lysine	127	127	127
Digestible methionine and cystine	65	65	65
Digestible threonine	73	73	73
Digestible tryptophan	21	21	21

lasted for two weeks.

At the end of the test period the piglets were anaesthetized with fluothane[R] (Hoechst GmBH, Munich, West Germany), nitrous oxide and oxygen. The abdomen was opened, and from three different places of the jejunal wall two tissue samples were taken: 0.5 m caudal to the ligament of Treitz, the middle of the jejunum, and 0.5 m proximal to the ileocecal ligament. The animals were subsequently euthanatized with T61[R] (Trofield surgicals Aktien Gesellschaft, Switzerland) intracardially.

From each jejunal part, one of the samples was cut longitudinally, spread out with the serosal side facing down, and affixed to dental wax plates with hedgehog spines. It was then fixed in a 4% buffered formalin solution for stereomicroscopic and histologic investigations. The other one was also cut open longitudinally, sealed in a plastic bag, and frozen (minus 70 °C) for biochemical determination of the activity of brush border enzymes. The biochemical investigation was limited to the control group and test group II.

The animals were weighed at the beginning of the test period, after 1 week, and at the end of the test period.

Stereomicroscopic studies

The shape of the intestinal villi was examined on the jejunal sample lying in a Petri dish just under the surface of the fixative. Fragments of ingesta and mucus, covering the villi, were carefully removed with a fine brush. The structure of the mucosa was then studied through a Zeiss dissecting microscope and graded according to Mouwen.[16] Depending on the degree of alteration of the intestinal villi a grading from 0 to 2½ was used (Table 3). Only the mucosa lying outside the areas with Peyer's patches was taken into consideration.

Table 3. Criteria for grading small intestinal mucosa by stereomicroscopy.

Grade	Criteria
0	A normal villous pattern with almost all finger-shaped villi.
½	mixed finger- and tongue-shaped villi.
1	A pattern with predominantly long to short tongue-shaped villi with few finger-shaped and leave-shaped ones.
1½	Predominantly short tongue- and leaf-shaped villi with few long tongue- and ridge-shaped villi.
2	Mixture of short tongue-, leaf-, and ridge-shaped and convoluted villi.
2½	Similar to grade 2 with flat areas.
3	Flat mucosa.

Morphometrical and histochemical studies

From the middle of each jejunal sample, a three millimeter wide zone was cut at right angles to the surface of the mucosa, carefully placed on its cut edge, and embedded in paraffin wax. Serial sections (5 μm) were cut and stained with hematoxylin and eosin and the periodic acid Schiff method.[15] Ten well-oriented villi and crypts per jejunal sample were measured at 100x magnification by means of a TEA Image Manager system (TEA and DIFA Measuring Systems, Breda, The Netherlands). The height of the villus was represented by the distance from the crypt opening to the tip of the villus. The crypt depth was determined from the base of the crypt to the level of the crypt opening. The villus/crypt ratio was calculated. The same crypt columns were used to determine the number of mitoses (meta- and anaphases). The mitotic activity was expressed as the number of mitoses per 100 crypt cells (index of mitosis).

Sections stained with periodic acid Schiff were used to count the number of goblet cells, in thirty crypts as well as in thirty villi.

As a means to access the functional state of the microvilli of the villous epithelium the activity of the brush border enzymes aminopeptidase and sucrase-isomaltase were determined. Brush border membranes were prepared according to the method of Hendriks *et al.*[7] The activity of the brush border enzymes was determined using the method of Bergmeyer[1] for aminopeptidase and sucrase-isomaltase. The activity was expressed in units per gram of brush border membrane protein. The amount of brush border membrane protein was determined according to Bradford[2].

Statistical analysis

Stereomicroscopic differences between control and test animals were evaluated for statistical significance using the Wilcoxon rank sum analysis. Statistical significance of the morphometric jejunal determinations, body weight data, and biochemical data between control and test animals was assessed using the analysis of variance plus comparison of means.

Results

Clinical findings

The body weight of the test animals (groups I and II) and control ones during the experimental period are summarized in Fig. 1. During the test period there was a serious growth inhibition and even weight loss in the test animals compared to the control ones. The differences were statistically significant ($P < 0.05$). No differences were found concerning the body weight between test groups I and II. All the test animals showed scouring during the

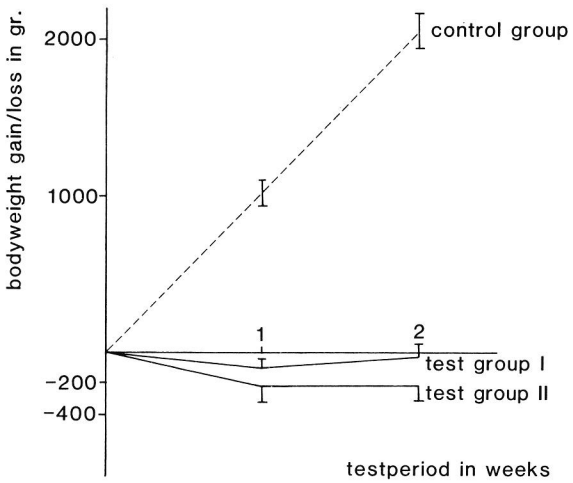

Figure 1. Body weight gain/loss in grams of the test animals (group I and II) and control group during the test period.

test period.

Stereomicroscopic features

The results of the stereomicroscopic investigation are summarized in Fig. 2. A distinct change in the three-dimensional villous structure of the test groups occurred when compared to the control ones. A shift took place in the villous shape of the test animals towards the higher gradations of the Mouwen classification. Compared to control villi (Fig. 3), predominantly abnormally shaped villi (tongue,- leaf-, or ridge-shaped and convoluted villi) were present in the test animals (Fig.4). There were even flat areas where the villi had completely disappeared. The most severe changes were present in the first and middle parts of the jejunum. Statistical calculations showed a significant difference between the control group ($P < 0.05$) and the test groups I and II (Fig. 2). No essential differences were found between the two test groups (Fig. 2). The surface of the mucosal samples from the test animals also appeared to be covered with more mucus than that of the control animals.

Histomorphometric features

The histology of the jejunal mucosa of a control and a test animal is depicted in Figs. 5 and 6, respectively.

The measured villus height, crypt depth, villus/crypt ratio, index of

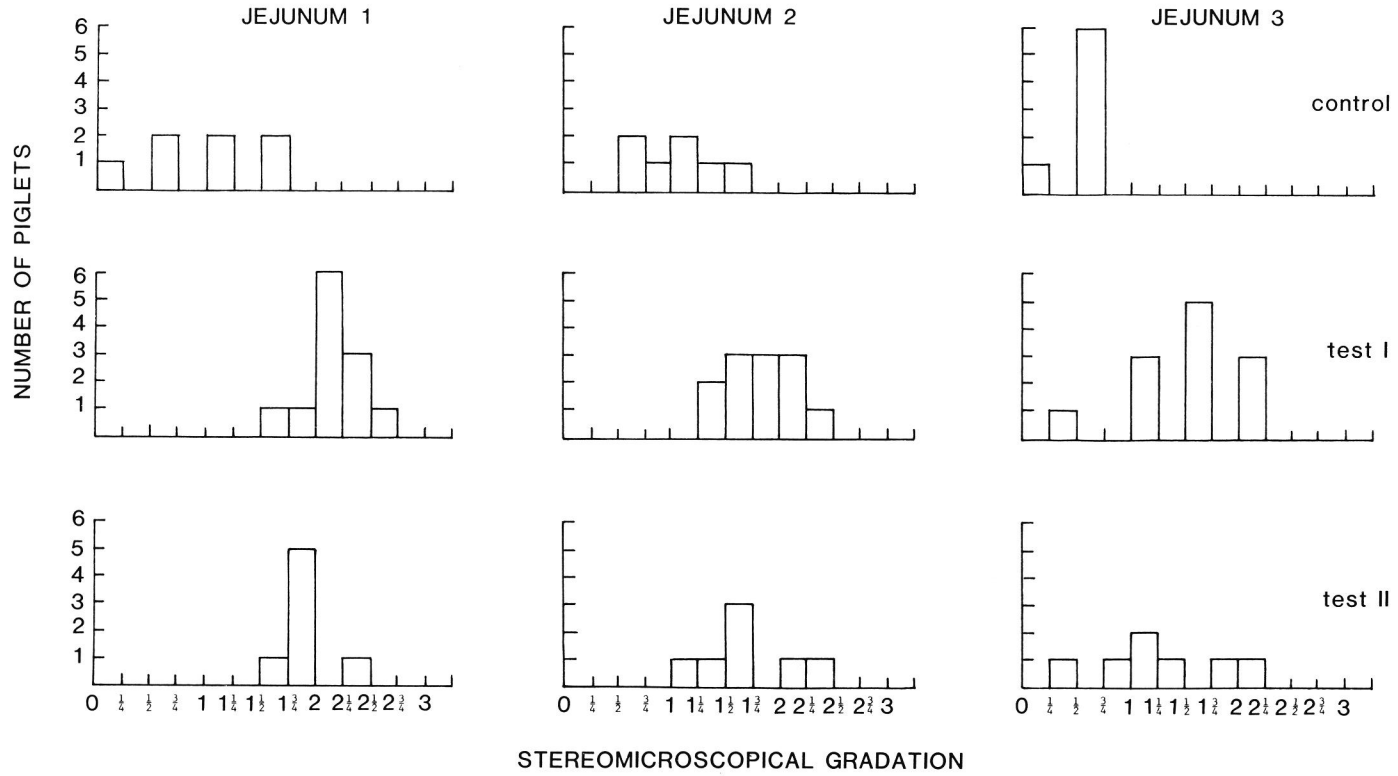

Figure 2. Mucosal pattern as graded by stereomicroscopy according to the Mouwen classification of the jejunum of control piglets and of piglets from the test groups (I and II).

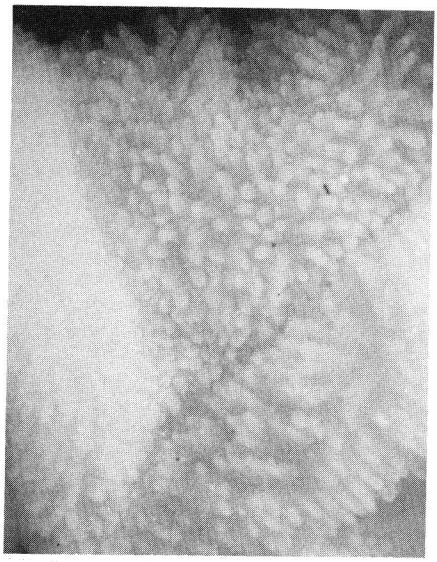

Figure 3. Jejunal mucosa; control piglet. Predominantly finger-shaped villi (F) are adjacent to tongue-shaped (T) ones.

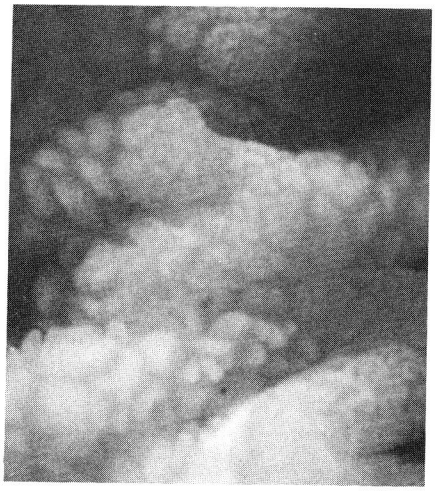

Figure 4. Jejunal mucosa; test piglet fed *Phaseolus vulgaris*. Note the mixture of tongue-(T), leaf-(L), and ridge-shaped (R) and convoluted (C) villi.

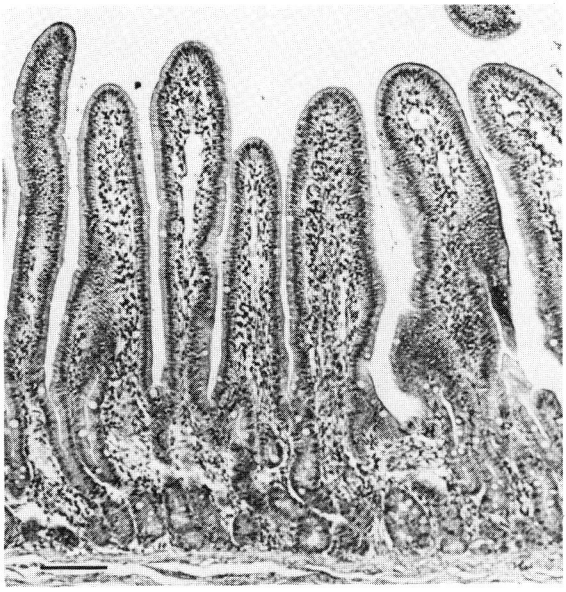

Figure 5. Jejunal mucosa; control piglet, HE. Bar = 100 μm.

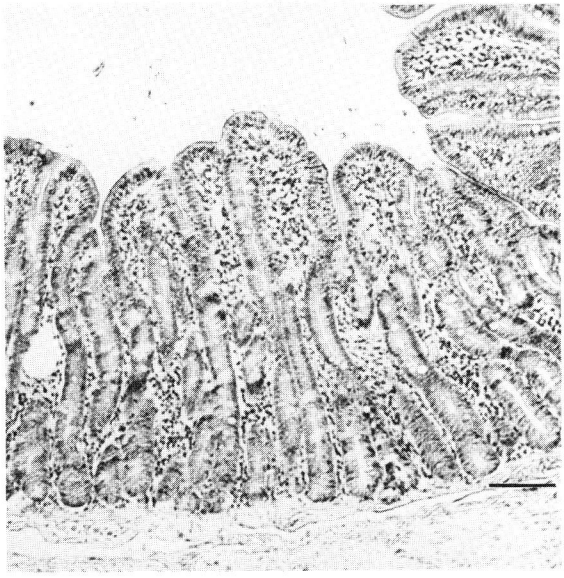

Figure 6. Jejunal mucosa; test piglet fed *Phaseolus vulgaris*. Note the shortening of the villi and lengthening of the crypts, HE. Bar = 100 μm.

Table 4. Histometric characteristics of the jejunal mucosa in the control and the test animals (group I and II)

	Part of the jejunum	Number of animals	Villus height (μm) ± SEM	Crypt depth (μm) ± SEM	Villus/crypt ratio ± SEM	Index of mitoses ± SEM	Goblet cells/ villus ± SEM	Goblet cells/ crypt ± SEM
control	1	7	314.2 ± 23,6	290,3 ± 10.3	1.1 ± 0.20	4.4 ± 1.0	11.2 ± 0.9	17.7 ± 1.7
	2	7	434.3 ± 42.7	236.2 ± 11.3	1.9 ± 0.70	7.1 ± 0.7	11.5 ± 1.7	15.1 ± 0.8
	3	7	392.7 ± 30.3	219.2 ± 9.7	1.8 ± 1.20	4,6 ± 1.2	13.3 ± 1.5	17.4 ± 0.8
test I	1	11	177.8 ± 13.5	331.3 ± 15.4	0.6 ± 0.04	18.8 ± 0.9	6.0 ± 0.7	23.6 ± 2.2
	2	11	246.6 ± 17.2	288.8 ± 8.9	0.9 ± 0.05	19.6 ± 0.9	6.2 ± 0.9	24.2 ± 1.5
	3	11	290.7 ± 24.3	269.4 ± 8.4	1.1 ± 0.08	18.5 ± 0.8	11.4 ± 1.4	24.6 ± 1.4
test II	1	7	163.0 ± 12.8	325.7 ± 10.4	0.5 ± 0.03	14.0 ± 1.2	4.8 ± 0.9	22.6 ± 2.3
	2	7	225.2 ± 18.6	305.7 ± 14.7	0.8 ± 0.09	15.1 ± 0.3	6.3 ± 1.0	20.3 ± 1.6
	3	7	261.4 ± 26.5	256.6 ± 19.6	1.2 ± 0.10	14.1 ± 0.9	9.9 ± 1.8	22.4 ± 2.0

mitosis and the number of goblet cells are summarized in Table 4. The height of the villi was drastically reduced in the test animals , whereas the depth of the crypts was increased. A significant reduction of the ratio of villus height to crypt depth was calculated for the test groups in comparison to the control group (Table 4). These differences were encountered in all jejunal parts sampled. The differences between each test and the control group were statistically significant ($P < 0.05$).

The index of mitosis was significantly higher in animals from the test groups ($P < 0.05$). The number of villus goblet cells of the test groups was lower than that of the control group, whereas this number was higher in the crypts of the test animals compared to the control ones (Table 4). The differences were statistically significant ($P < 0.05$). The most severe changes were found in the first and middle parts of the jejunum. No essential differences were found between the test groups I and II.

Biochemical features

The results of the biochemical investigations are summarized in Table 5. The activities of amino-peptidase and sucrase-isomaltase were lower in the test animals (group II). These differences were statistically significant ($P < 0.05$).

Table 5. Activities of aminopeptidase and sucrase-isomaltase in U/g of brush-border membrane protein in the jejunal samples from control and test animals (group II).

Piglets	Aminopeptidase U/g of protein mean ± SEM	Sucrase-isomaltase U/g of protein mean ± SEM
control	2805.4 ± 419,1	835.4 ± 121.0
Number of samples	20	20
test group II	1678.7*± 115.5	378.1*± 58.1
Number of samples	20	20

* significantly different ($P < 0.05$) from controls.

Discussion

The stereomicroscopic and histomorphometric findings indicate that the structure of the jejunal mucosa of piglets fed a diet containing *P. vulgaris* beans differs from that of the piglets fed the control diet. However, no essential differences could be found between the two different test diets used. Stereomicroscopically, a shift took place in the villous shape of the test animals towards the higher gradations of the Mouwen classification. Histomorphometrically the jejunal mucosa of the test animals was characterized by shortening of the villi, lengthening of the crypts and increased mitotic activity. The most severe of these mucosal changes were found in the cranial part of the jejunum. The histomorphometric changes of villus atrophy and elongation of crypts are in agreement with similar findings in rats[13,23]. King *et al.*[8,9] however, could not observe a change in villus length in either rats or piglets fed *P. vulgaris* beans. On the other hand, Pusztai *et al.*[22] noted an increase in villus length in rats fed diets containing *P. vulgaris* beans.

The smaller number of goblet cells found on the villi compared to those in the crypts of the test animals is probably indicative of excessive stimulation and depletion of these cells. The presence of a larger amount of mucus on the mucosal surface of the test animals, as also described in rats[5], is compatible with this interpretation.

The reduction of the activities of amino-peptidase and sucrase-isomaltase in the brush border of the test animals may be due to direct inhibition of these enzymes through cell surface-*P. vulgaris* agglutinin interaction[14], or to immaturity of the villous epithelium, due to the villus atrophy[10,24].

The changes of the jejunal mucosa may be caused by the lectins present in the unheated *P. vulgaris* beans[21,23].

The morphologic and functional changes of the small intestinal mucosa may result in a reduction of the digestive and absorptive capacity, accounting for the diminished growth, weight loss and diarrhea of the test animals. We conclude that severe morphologic and biochemical mucosal changes of the small intestine occur after feeding of *P. vulgaris* beans. Furthermore, the pig fed raw *P. vulgaris* beans may provide a useful model in which way antinutritional factors react with the intestinal mucosa.

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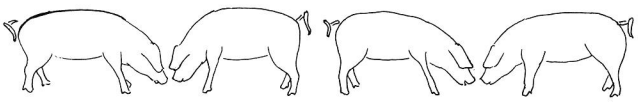
CHAPTER IV

BINDING OF KIDNEY BEAN (*PHASEOLUS VULGARIS*) ISOLECTINS TO DIFFERENTIATED HUMAN COLON CARCINOMA CACO-2 CELLS AND THEIR EFFECTS ON CELLULAR METABOLISM



HGCJM Hendriks, Maria JL Kik, JFJG Koninkx, TSGAM van den Ingh, and JMVM Mouwen

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Summary

In this investigation we have studied the binding of PHA-isolectins L_4 and E_4 to the brush-border membrane of differentiated Caco-2 cells and assessed the impact on the cellular metabolism and the microvilli.

Computer analysis of the data based on binding experiments with peroxidase-conjugated isolectins revealed values for maximal binding of $(2540 \pm 151).10^{-9}$ M PHA-L4 and $(2104 \pm 140).10^{-9}$ M PHA-E4 per mg of brush-border membrane protein. The dissociation constant for L_4 and E_4 binding was $(4.3 \pm 1.4).10^{-6}$ M and $(1.1 \pm 0.8).10^{-6}$ M, respectively.

Incubation of differentiated Caco-2 cells for 30 min. with ferritin-conjugated PHA-isolectins revealed that, as indicated by the number of ferritin particles, PHA-E4 bound to the microvilli to a greater extent than PHA-L4. Ferritin particles were also localized intracellularly over endocytotic invaginations and vesicles.

After incubation for 48 hours with PHA-L4 or PHA-E4 the relative incorporation of precursors for DNA-, RNA- and (glyco)protein synthesis into the trichloro-acetic acid insoluble fraction of the Caco-2 cells was determined. Both isolectins stimulated the incorporation of thymidine and glucosamine, whereas neither PHA-L4 nor PHA-E4 were able to influence the incorporation of uridine. With respect to fucose, methionine and N-acetyl mannosamine the stimulatory effect remained confined to PHA-E4. Since PHA-L4 and PHA-E4 were tested at the same concentration, PHA-E4 is more effective than PHA-L4. The changes in the uptake of radioactive precursors were lost after heat-inactivation of PHA-E4.

Compared to control and PHA-L4 incubated Caco-2 cells the microvilli of PHA-E4 incubated cells were shortened significantly ($p < 0.01$).

Introduction

Lectins of plant origin, mostly present in food elements, sometimes induce severe damage to the intestinal tract, unless their activity is destroyed by heating[9,10,14,15,18]. In addition, lectins are known to interfere with cellular processes in different types of cells[16]. Essentially all metabolic processes examined in *Phaseolus vulgaris* agglutinin-treated lymphocytes are stimulated though to varying degrees, and at different times after exposure to the mitogen[8]. There are relatively few studies on lectins in relation to the cellular metabolism in enterocytes[1,4,6,19,21,24]. After binding of the dietary lectins through their sugar-reactive sites to the enterocytes and/or the subsequent endocytosis of the lectin-receptor complex[11], disruption of the structure of the brush-border takes place *in vivo*[10,11] as well as *in vitro*[5].

Recently we have demonstrated that, because of the similarity between the lectin-induced lesions *in vivo* and *in vitro*, the human colon carcinoma cell line Caco-2 might represent a suitable model for investigations on the multifold aspects of lectins in differentiated enterocytes[5].

In the experiments presented in this study we have used differentiated Caco-2 cells, which display both structural (microvilli, tight junctions) and functional (brush-border enzymes) characteristics of small intestinal enterocytes[23], to investigate the binding of PHA-isolectins L_4 and E_4 to these cells and the subsequent lectin-induced effects on the microvilli and the cellular metabolism. In particular we have examined the effects of both isolectins on the DNA-, RNA- and (glyco)protein synthesis using several specific radioactive precursors.

Materials and Methods

Caco-2 cell culture.

The Caco-2 cells were grown in Dulbecco's modified Eagle medium (DMEM) (Flow Laboratories, Amstelstad B.V., Amsterdam, The Netherlands) supplemented with 1% (v/v) non-essential amino acids (Flow), 50 μg of gentamycin/ml (Flow), 10 mM sodium bicarbonate (Flow), 25 mM Hepes (Flow) and 20% (v/v) fetal calf serum (FCS) (Sanbio B.V., Uden, The Netherlands) and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were seeded at 4.10^4 cells/cm^2 using tissue culture 24 flat bottom well plates (2 cm^2/well) (Gibco Europe B.V., Hoofddorp, The Netherlands) or 75 cm^2 tissue culture flasks (Costar Europe Ltd, Badhoevedorp, The Netherlands) containing 1 or 15 ml of culture medium respectively. The culture medium was changed 3 times a week. This study encompassed 32 passages of the cell line ranging from the 69th to the 101th.

Since incubation of the differentiated Caco-2 cells with PHA-isolectins should be carried out in medium lacking any carbohydrate sources, the percentage of

FCS was gradually reduced, whereas at the same time the amount of the serum substitute Ultroser G (Reactifs IBF, LKB-Products B.V., Zoetermeer, The Netherlands) was increased. On day 14 after cell-seeding the culture medium containing 20% (v/v) FCS was replaced by 1 ml of culture medium containing 10% (v/v) FCS and 1% (v/v) Ultroser G, on day 16 by 1 ml of culture medium containing 1% (v/v) FCS and 2% (v/v) Ultroser G and on day 19 by 0.5 ml of DMEM.

Incubation of differentiated Caco-2 cells by PHA-isolectins L_4 and E_4.

Incubation of the cells was performed in quadruplicate in 0.5 ml of DMEM containing 0, 40, 80, 120, 160 and 200 μg of PHA-L4/ml or PHA-E4/ml (E-Y Laboratories Inc., San Mateo, California, U.S.A.). For concentrations lower than 500 μg/ml, PHA-L4 shows no agglutination with human erythrocytes, whereas PHA-E4 at a concentration less than 20 μg/ml agglutinates 3% human erythrocytes (product information).

After incubation for 44 hours 100 μl of DMEM containing ^{14}C-thymidine* (0.02 μCi) and ^{3}H-glucosamine* (0.8 μCi), ^{14}C-uridine* (0.02 μCi) and ^{3}H-fucose* (2μCi) or ^{35}S-methionine* (0.1 μCi) and N-acetyl ^{3}H-mannosamine** (3 μCi) was added to the monolayer and double-labeling incubation was continued for 4 hours (*Amersham Nederland B.V., Houten, The Netherlands; **New England Nuclear, Du Pont de Nemours B.V., 's-Hertogenbosch, The Netherlands). The selection of the radioactive monosaccharides was made on the basis of their entry point in the metabolic pathway and the metabolic conversion to other monosaccharides[3]. The incorporation was stopped by adding 1 ml of 10% trichloro-acetic acid (0°C) to the monolayer. Subsequently the monolayer was washed 2 times with 1 ml of 0.01 M PBS (0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, 0.9% NaCl), pH = 7.3, fixed with 1 ml of methanol during 10 min. and dissolved in 0.5 ml of 0.1 N NaOH. The incorporated radioactivity present in 0.2 ml of 0.1 N NaOH was determined by liquid scintillation counting in 2 ml of Dynagel™ (J.T. Baker Chemical B.V., Deventer, The Netherlands) as scintillant and performed with a Beckman LS 1701 (Beckman Instruments B.V., Mijdrecht, The Netherlands).

The incorporated radioactivity was calculated as d.p.m./μg of DNA[12] and expressed as the relative incorporation (factor by which the incorporation was increased or decreased as compared to cell cultures which were not incubated with lectins).

The effect of the lectin concentrations on cell viability was determined by trypan blue exclusion.

Preparation of purified brush-border membranes from differentiated Caco-2 cells.

After washing the cell monolayer 3 times with 5 ml of 0.01 M PBS, pH = 7.3 at 37°C, the cells were removed from the surface of the culture flasks after incubation during 45 min. at 37°C with 5 ml of transfer medium (8.0 g NaCl, 0.2 g KH_2PO_4 and 0.2 g $Na_2EDTA \cdot 2H_2O$/l). The resulting cell pellet formed by centrifugation (10 min. 500 g) at 4°C was washed and pelleted 3 times with 10 ml of 0.01 M PBS, pH = 7.3 (4°C) and finally stored at -70°C. Highly purified brush-border membranes were prepared according to a modification of an existing method[26]. Briefly, the isolation procedure performed at 4°C consists of the following steps: homogenization and ultrasonication in 2 mM Tris-50 mM mannitol buffer, pH = 7.1; Ca^{2+}-treatment of the homogenate; centrifugation (10 min. at 950 g and 30 min. at 33.500 g); Tris disruption of brush border fragments; purification of the brush-border membranes by gradient-ultracentrifugation (15 min. at 63.000 g); pelleting by centrifugation (60 min. at 200.000 g) of the membrane fraction formed at the interface between 37% and 42% glycerol. The modification applied to the glycerol gradient. The gradient was composed of 2 layers: 1.5 ml 37% glycerol and 5.5 ml 42% glycerol in a separation medium of 0.05 M $MgCl_2$.

Binding of peroxidase-conjugated *Phaseolus vulgaris* isolectins to the brush-border membranes of differentiated Caco-2 cells.

Purified brush-border membranes (200.000 g pellet)[26] were used to demonstrate the binding of the PHA-isolectins L_4 and E_4. The lectin binding to the brush-border membranes isolated from differentiated Caco-2 cells has been determined quantitatively using the enzyme-linked lectin sorbent assay (ELLSA)[7].

By means of a ligand program the specific binding was calculated in saturation experiments from the untransformed total binding data and from the specific binding data using corrected free ligand concentrations. The data were fitted in a one binding site and a two binding sites model. The best fit (one binding site model) was chosen using least-squares values that were statistically compared in a F-test.
Inhibition studies were conducted using excess lectin specific monosaccharide. The non-specific binding in this assay approximated 30% of the total binding.

Binding of ferritin-conjugated *Phaseolus vulgaris* isolectins to differentiated Caco-2 cells.

Differentiated Caco-2 cells were grown in tissue culture 24 flat bottom well plates on Thermanox™ tissue culture cover slips (Flow). After three washes with

5 ml of 0.01 M PBS, pH = 7.35 the cell monolayers were incubated at 37°C for 30 min. with 50 μg of PHA-L4 ferritin/ml or PHA-E4 ferritin/ml in 0.01 M PBS, pH = 7.35. Subsequently the cells were rinsed 3 times with 1 ml of 0.01 M PBS, pH = 7.35 and submitted to electronmicroscopic fixation and embedding procedures. Following pre-fixation for 120 min. with 0.5% (v/v) glutaraldehyde in 0.1 M phosphate-buffer, pH = 7.35 (4°C), postfixation for 30 min. with 2% (v/v) OsO_4 in 0.1 M phosphate-buffer, pH = 7.35 (4°C) and dehydration the cell monolayer was embedded in Araldite. Fixation and embedding of the cell monolayers were performed in situ[17,23].

Morphological changes in the microvilli of differentiated Caco-2 cells after incubation with PHA-isolectins.

Differentiated Caco-2 cells grown in tissue culture 24 flat bottom well plates on Thermanox™ tissue culture cover slips were incubated at 37°C for 48 hours with 50 μg of PHA-L4/ml or PHA-E4/ml in 0.5 ml of DMEM. Post-incubation and subsequent electronmicroscopic fixation and embedding procedures were as described in the above-mentioned section.

Statistical significance between the length of microvilli of control and PHA-isolectin incubated Caco-2 cells was assessed using an analysis of variance plus comparison of means. Statistical significance was accepted at the $p < 0.05$ level.

Results

Binding of *Phaseolus vulgaris* isolectins L_4 and E_4 to the differentiated Caco-2 cells and their isolated brush-border membrane fraction.

The specific binding of PHA-L4 and PHA-E4 to the brush-border membranes of differentiated Caco-2 cells is demonstrated in Figure 1.

Computer analysis of the individual curves by means of iterized computer fit analysis of the data revealed values for maximal binding per mg of brush-border membrane protein and dissociation constants for both isolectins (Table 1).

The brush-border membrane fraction had slightly more PHA-L4 than PHA-E4 binding sites per mg of brush-border membrane protein. Compared to PHA-E4, the dissociation constant of PHA-L4 was 4 times greater. These data suggest that both PHA-L4 and PHA-E4 are able to interact directly with the microvillous membranes of differentiated enterocyte-like Caco-2 cells.

Extracellular labelling with ferritin-conjugated *P. vulgaris* isolectins L_4 and E_4, as indicated by ferritin particles, was evident on the surface of the Caco-2 cells (Fig. 2). PHA-E4 binds to the microvilli of the Caco-2 cells to a greater extent than PHA-L4. However, between the distribution of the ferritin particles no differences could be detected. The ferritin particles are located almost

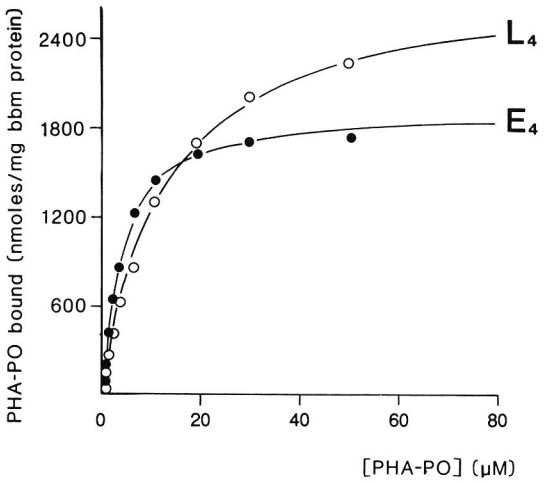

Figure 1. Specific binding of peroxidase-conjugated PHA-L_4 and PHA-E_4 to purified brush-border membranes of differentiated Caco-2 cells.

These binding curves have been used for calculation of maximal lectin binding and dissociation constant. Binding studies were carried out as described previously[20]. Abbreviations: bbm: brush-border membrane; PHA-PO: peroxidase-conjugated *Phaseolus vulgaris* agglutinin.

Table 1. Binding of peroxidase-conjugated PHA-L_4 and PHA-E_4 to purified brush-border membranes of differentiated Caco-2 cells.

PHA-isolectin	Lectin binding/mg of brush-border membrane protein (M)	Dissociation constant (M)
L_4	$(2540 \pm 151).10^{-9}$	$(4.3 \pm 1.4).10^{-6}$
E_4	$(2104 \pm 140).10^{-9}$	$(1.1 \pm 0.8).10^{-6}$

The number of *Phaseolus vulgaris* isolectin binding sites in the brush-border membrane has been established by means of iterized computer fit analysis of the data[20]. The results are expressed as the mean maximal binding ± SD and the mean dissociation constant ± SD of two different passages.

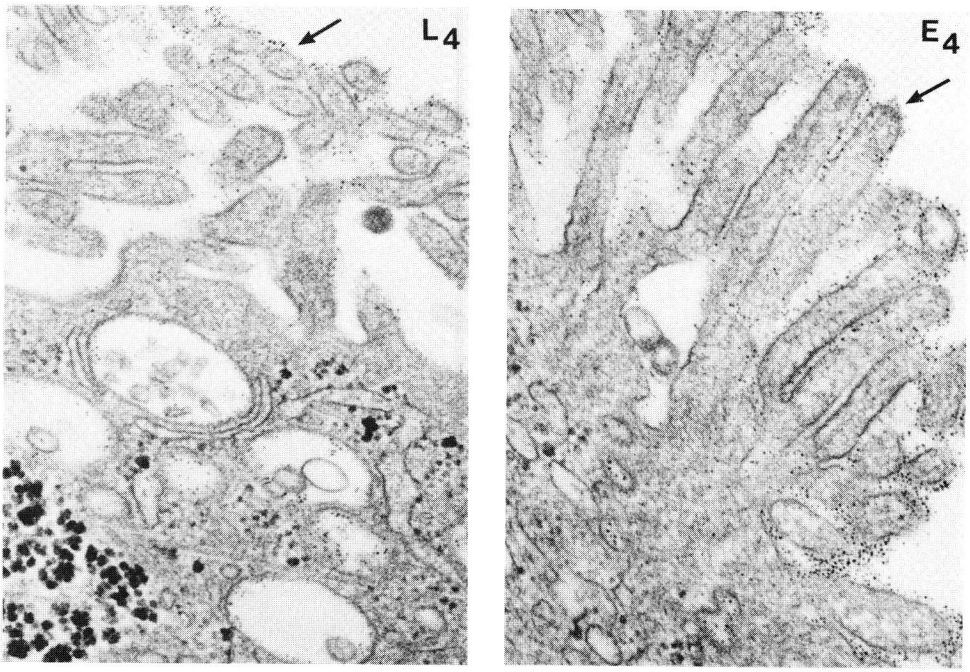

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Figure 2. Binding of ferritin-conjugated PHA-L_4 and PHA-E_4 to the microvilli of differentiated Caco-2 cells.

In comparison with PHA-E_4 (a) the binding of PHA-L_4 (b) is less evident. (arrows) Very few ferritin particles are present over the cores of the microvilli. Note the presence of ferritin-particles in apical vesicles (arrow heads) (x 63.050).

exclusively over the glycocalyx of the microvilli. Only few ferritin particles are present over the cores of the microvilli.
Intracellular labelling was present in endocytotic vesicles over the apical cytoplasm of the Caco-2 cells (Fig. 2).

Incorporation of precursors for DNA-, RNA- and (glyco)protein synthesis by differentiated Caco-2 cells after incubation with PHA-L4 and PHA-E4.

When PHA-L4 was added to the culture medium and incubation was performed for 48 hours, the relative incorporation of ^{14}C-thymidine and ^{3}H-glucosamine appeared to increase with increasing lectin concentrations, whereas no change in the relative incorporation of ^{14}C-uridine, ^{35}S-methionine, ^{3}H-fucose and N-acetyl ^{3}H-mannosamine could be observed (Fig. 3). In comparison with PHA-L4 the stimulatory effect expressed as the relative incorporation of ^{14}C-

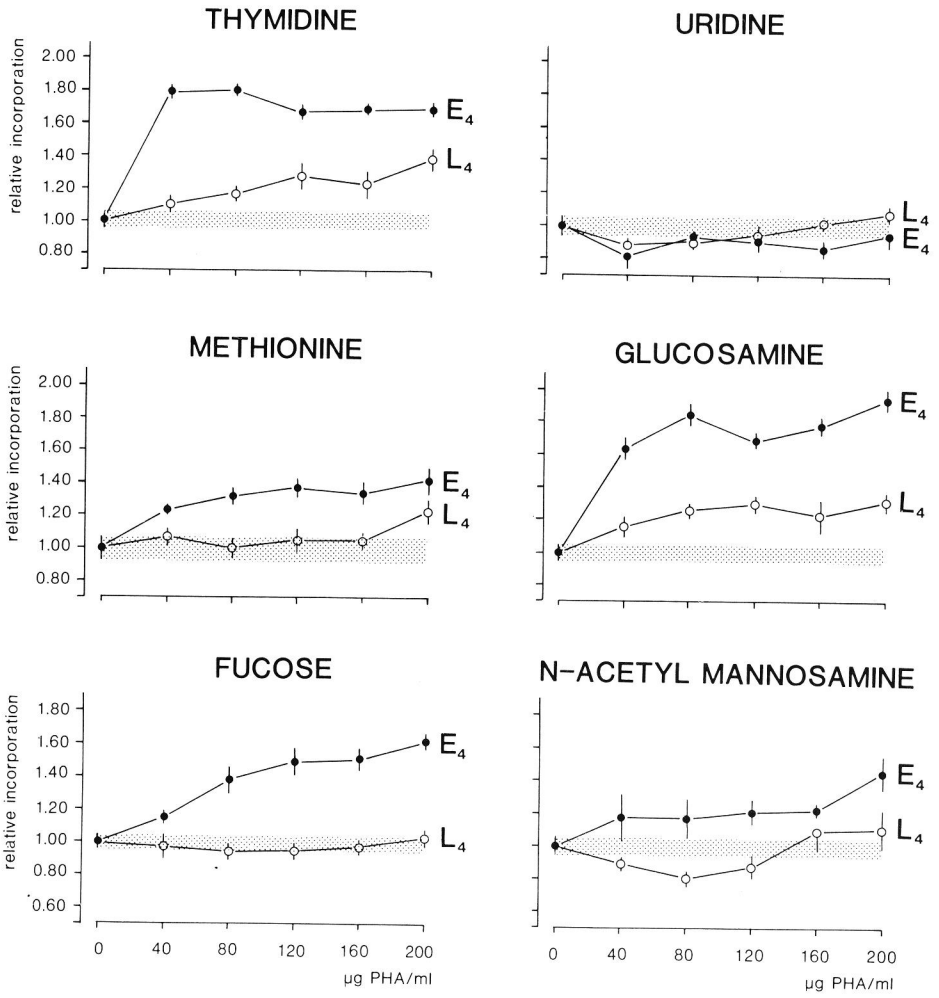


Figure 3. Relative incorporation of ^{14}C-thymidine, ^{14}C-uridine, ^{35}S-methionine, ^{3}H-glucosamine, ^{3}H-fucose and N-acetyl ^{3}H-manno-samine by differentiated Caco-2 cells following incubation with PHA-L_4 or PHA-E_4.

Two different passages have been used to establish the effect of PHA-L_4 and PHA-E_4. The results are expressed as the mean relative incorporation ± SEM. The dotted area represents the mean relative incorporation ± SEM of cell cultures which are not incubated with lectins. For each passage of the cells the relative incorporation of all lectin concentrations was determined using quadruplicate cultures.

thymidine and ^{3}H-glucosamine appeared to be significantly higher for PHA-E4. Moreover, PHA-E4 concentrations as low as 40 μg/ml already displayed a maximal stimulatory effect with respect to the relative incorporation of ^{14}C-thymidine, whereas at the same lectin concentration the relative incorporation of ^{3}H-glucosamine of PHA-E4 incubated cells was 85% greater than in control cells (Fig. 3).

As opposed to PHA-L4 a marked increase of the relative incorporation of both ^{35}S-methionine and ^{3}H-fucose was observed after incubation with PHA-E4. The increase in the relative incorporation of N-acetyl ^{3}H-mannosamine was only slightly. The relative incorporation of ^{14}C-uridine did not change irrespective of the PHA-isolectin concentration.

The changes in the relative incorporation of ^{14}C-thymidine, ^{35}S-methionine, ^{3}H-glucosamine, ^{3}H-fucose and N-acetyl ^{3}H-mannosamine as demonstrated in Figure 3 vanished, when the differentiated Caco-2 cells were incubated with 200 μg of heat-inactivated PHA-E4/ml (15 min. at 100°C) (Table 2).

Irrespective of the isolectin and lectin concentration tested cell viability determined by trypan blue exclusion was $\geq$ 95%. Also the DNA-content per well (mean $\pm$ SD) appeared to be independent of the isolectin as well as lectin concentration tested (64.9 $\pm$ 3.9 μg DNA/well for 0-200 μg of PHA-E4/ml and 62.2 $\pm$ 4.1 μg DNA/well for 0-200 μg of PHA-L4/ml).

Table 2. The effect of heat-inactivated PHA-E_4 upon the relative incorporation of radioactive precursors by differentiated Caco-2 cells.

Precursor	Relative incorporation	
	No heat-treatment of PHA-E_4	Heat-inactivated PHA-E_4
^{14}C-thymidine	1.71 $\pm$ 0.04	1.03 $\pm$ 0.06
^{14}C-uridine	0.95 $\pm$ 0.07	1.05 $\pm$ 0.07
^{35}S-methionine	1.45 $\pm$ 0.02	1.00 $\pm$ 0.03
^{3}H-glucosamine	1.96 $\pm$ 0.09	1.02 $\pm$ 0.05
^{3}H-fucose	1.62 $\pm$ 0.04	0.99 $\pm$ 0.05
N-acetyl ^{3}H-mannosamine	1.45 $\pm$ 0.10	1.01 $\pm$ 0.06

Two different passages have been used to establish the effect of heating. The results are expressed as the mean relative incorporation $\pm$ SEM. For each passage of the cells the relative incorporation of PHA-E_4 and heat-treated PHA-E_4 (200 μg/ml) was determined using quadruplicate cultures.

Ultrastructural changes in the microvilli of differentiated Caco-2 cells after incubation with PHA-L4 and PHA-E4.

Differentiated Caco-2 cells have been exposed for 48 hours to 50 μg of PHA-L4/ml or 50 μg of PHA-E4/ml. The ultrastructural morphology of the microvilli of these cells as well as control cells is demonstrated in Figure 4.

In comparison with control Caco-2 cells and PHA-L4 incubated cells the microvilli of PHA-E4 incubated cells clearly exhibited the presence of shortened microvilli (Fig. 4). A morphometric analysis revealed that the length of the microvilli of PHA-E4 incubated cells differed significantly ($p < 0.01$) from control cells (Table 3). The length of the microvilli of PHA-L4 incubated cells and control cells did not differ significantly.

The brush-borders of both PHA-L4 and PHA-E4 incubated Caco-2 cells contained membrane vesicles associated with the brush-border (Fig. 4). The brush-borders of control Caco-2 cells did not exhibit these membrane vesicles.

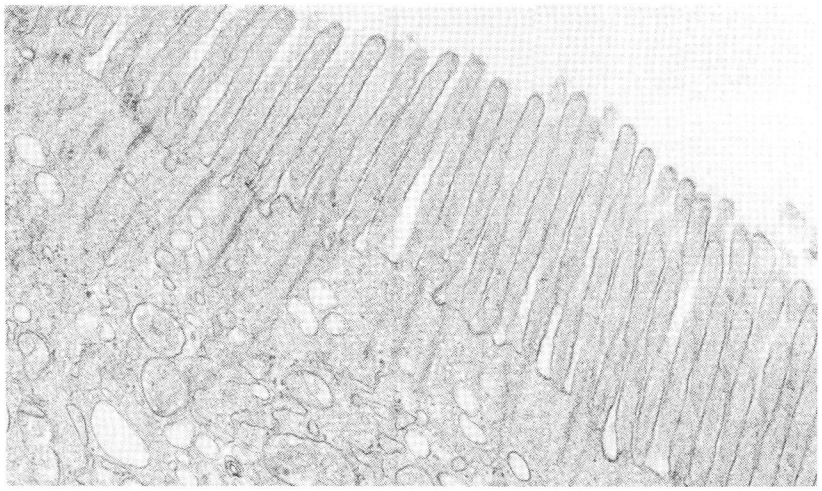
Table 3. Changes in microvillus length of differentiated Caco-2 cells after incubation with PHA-isolectins L_4 and E_4.

Caco-2 cells	Length of the microvilli (μm)
Controls	2.01 $\pm$ 0.05
PHA-L_4 incubated	1.91 $\pm$ 0.06
PHA-E_4 incubated	1.35 $\pm$ 0.04

Three different passages were used to measure the isolectin-induced alterations of the microvilli. Differentiated Caco-2 cells were incubated for 24 hours with 50 μg of PHA-L_4/ml or 50 μg of PHA-E_4/ml. Microvillus length between control cells and PHA-E_4 incubated cells differed significantly ($p < 0.01$). No significant difference was found between control cells and PHA-L_4 incubated cells. The results are expressed as the mean $\pm$ SEM.

Discussion

One of the objectives of the present study was to investigate the binding of the PHA-isolectins L_4 and E_4 to isolated brush-border membranes of Caco-2 cells and intact Caco-2 cells. The quantitative data of the binding of peroxidase-conjugated isolectins to the brush-border membranes are presented in Table 1. Substantial differences between the dissociation constant of PHA-L4 and PHA-E4 were determined. PHA-E4, the erythroagglutinating isolectin recognizes biantennary oligosaccharides, whereas PHA-L4, the leukoagglutinating isolectin



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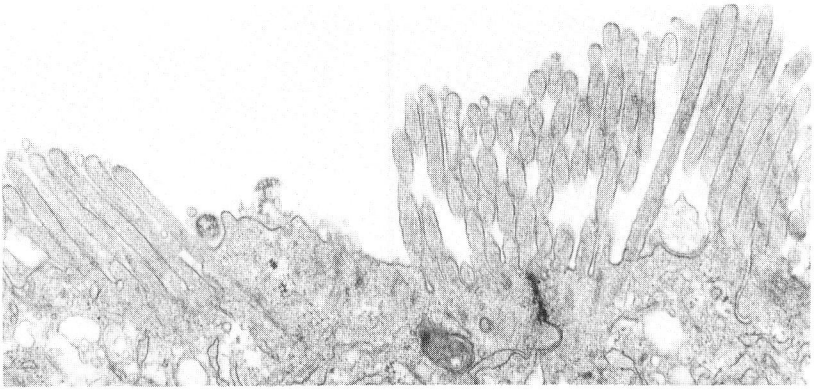

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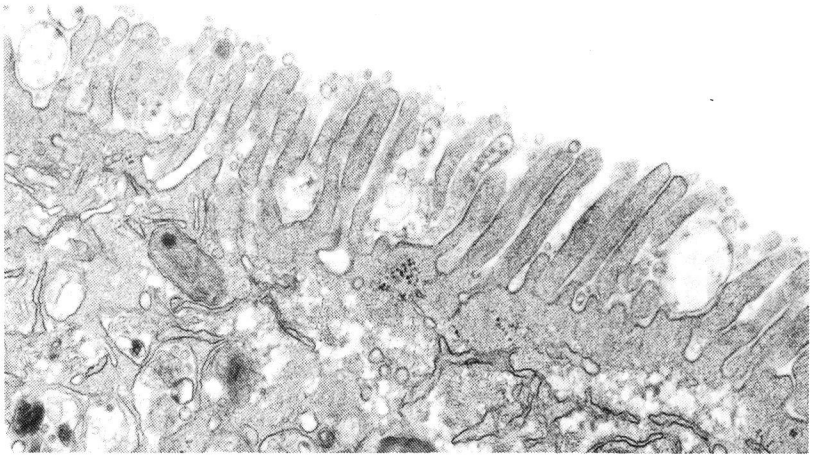

4C

binds specifically to tri- and tetra-antennary oligosaccharides[17]. This variety in oligosaccharide specificity between PHA-L4 and PHA-E4 appears to be expressed in the differences of the dissociation constants. In comparison with PHA-E4 the dissociation constant of PHA-L4 for isolated brush-border membranes of differentiated Caco-2 cells is four times greater. Binding studies in the literature report higher values for the association constants of the lectins (reciprocal value of dissociation constants). Association constants of $12.6 \cdot 10^6$ M^{-1} for PHA-L4 and $2.5 \cdot 10^6$ M^{-1} for PHA-E4 are calculated for the binding to purified rat brush-border membranes[2]. The different cell types from which brush-border membranes were isolated might explain these different values to a great extent.

By electron microscope immunocytochemistry immunogold localization of ingested PHA-L4 and PHA-E4 was demonstrated on the duodenal and jejunal microvilli and endocytosed into the lysosomal pathways of the rat enterocytes[11]. Recently direct experimental evidence has been presented demonstrating transepithelial transport and uptake of dietary PHA into the systemic circulation of the rat[25]. Also our binding experiments revealed that after incubation for 30 min. with ferritin-conjugated isolectins ferritin particles were present extracellularly on the microvilli of Caco-2 cells and intracellularly over apical invaginations and vesicles (Fig. 2). The short-time exposure of Caco-2 cells to PHA-L4 or PHA-E4 together with the different dissociation constants may explain the variety in binding of the isolectins.

In *in vivo* studies the incorporation of precursors in enterocytes is dependent on a direct action of the lectin on enterocyte metabolism as well as on an indirect effect mediated through the reduction of food intake[13]. In this *in vitro* study the direct interference of PHA-isolectins with the cellular metabolism of differentiated Caco-2 cells can be investigated. To detect any differences in their effect upon the cellular metabolism PHA-L4 and PHA-E4 were tested separately. Since incubation was performed with the very same concentrations, the results clearly demonstrate, that PHA-E4 stimulates the incorporation of ^{14}C-thymidine, ^{35}S-methionine, ^{3}H-glucosamine, ^{3}H-fucose and N-acetyl ^{3}H-mannosamine to a greater extent than PHA-L4 (Fig. 3; Table 2). After incubation of differentiated Caco-2 cells with heat-inactivated PHA-E4 (15 min. at 100°C), the stimulatory effect of PHA-E4, resulting in an increased incorporation of radioactive precursors, disappears (Table 2). This finding indicates that the stimulatory

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Figure 4. Microvilli of differentiated Caco-2 cells after incubation with PHA-L_4 and PHA-E_4.

Microvilli of control Caco-2 cells (a). Microvilli of Caco-2 cells after incubation for 48 hours with 50 μg of PHA-L_4/ml (b) or 50 μg of PHA-E_4/ml (c) (x 32.500).

effect was related to PHA-E4 binding to the specific biantennary oligosaccharide[17] on the cell surface membrane of differentiated Caco-2 cells.

Prolonged exposure of the rat intestinal cells to PHA is known to result in stimulation of the cellular metabolism[6,19,21,24]. Also, our incorporation experiments suggest that exposure for 48 hours to PHA-E4 results in stimulation of DNA- and (glyco)protein synthesis in differentiated Caco-2 cells. However, in contrast to another investigation[24], neither PHA-L4 nor PHA-E4, is able to enhance the incorporation of ^{14}C-uridine in Caco-2 cells. The DNA-content per well does not change significantly with increasing lectin concentration. Moreover, cell viability remains rather constant ($\geq$ 95%). For that reason DNA-synthesis most probably does not account for the increased incorporation of ^{14}C-thymidine. Whether the incorporation is due to an increased DNA repair is presently unknown.

In the presence of PHA-E4 the relative incorporation of ^{35}S-methionine, ^{3}H-glucosamine, ^{3}H-fucose and N-acetyl ^{3}H-mannosamine (Fig. 3; Table 2) increases, reflecting sustained synthesis of (glyco)proteins. At the same time the relative incorporation of ^{14}C-uridine does not change as compared to control Caco-2 cells. Therefore the data presented here suggest that the increased rate of synthesis of (glyco)proteins is due to a translational rather than transcriptional control of (glyco)protein synthesis. In contrast to *Ricinus communis* agglutinin (ricin and abrin), which after endocytosis inactivates the 60S ribosomal units[20], PHA-E4 might speed up (glyco)protein synthesis by assembling functional polysomes, increasing the net rate of initiation of protein synthesis or accelerating the translation rate.

The data presented here (Fig. 3) further indicate, that the PHA-L4 induced changes in the cellular metabolism do not correlate with the agglutination activity of this isolectin. PHA-L4 which does not possess any haemagglutinating activity at concentrations lower than 500 μg/ml, clearly acts upon the incorporation of ^{14}C-thymidine and ^{3}H-glucosamine by differentiated Caco-2 cells.

The results of several experiments have shown that exposure of rat intestinal epithelium to concanavalin A, wheat germ agglutinin and kidney bean (*P. vulgaris*) lectin causes disarrangement of the cytoskeleton of rat enterocytes[9,10,18,27]. Recently actin cytoskeletal lesions resulting in shortened microvilli have been found in differentiated Caco-2 cells after exposure to soybean agglutinin[5]. Shortening of the microvilli appeared to be accomplished by a shift in the ratio of globular: filamentous actin. As a result of exposure for 48 hours of differentiated Caco-2 cells to PHA-E4 the length of the microvilli of these cells were shortened significantly ($p < 0.01$) in comparison with control and PHA-L4 incubated cells (Fig. 4 and Table 3). It is therefore tempting to suggest that the PHA-E4 induced shortening of the length of the microvilli has to be attributed to actin cytoskeletal lesions as well. After exposure of rat intestinal epithelium to intraluminal dietary lectins clusters of vesicles associated with the brush-border can be found[10,18].

Also *in vitro* an increased number of vesicles nearby the brush-borders of PHA-

L4 and PHA-E4 incubated Caco-2 cells was observed (Fig. 4). The presence of these vesicles might reflect an increased turnover of the microvillous membrane.

After binding of the PHA-isolectins to the brush-border membrane receptors of differentiated Caco-2 cells and internalization of bound lectin, PHA-L4 as well as PHA-E4 induce a number of cell metabolic changes within the cells. The precise mechanism responsible for the stimulation of the cellular metabolism has not been clarified. Further experiments are necessary to elucidate the nature of the regulatory mechanisms of these events.

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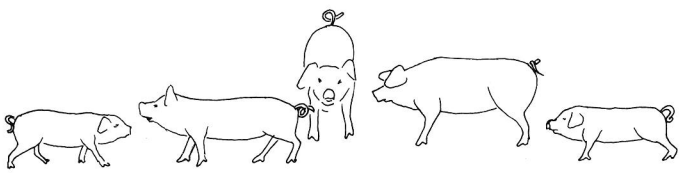
CHAPTER V

PATHOLOGICAL EFFECTS OF *PHASEOLUS VULGARIS* ISOLECTINS ON PIG JEJUNAL MUCOSA IN ORGAN CULTURE



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Abstract

The interaction of plant lectins with pig small intestinal epithelium in organ culture was studied.

The binding of *Phaseolus vulgaris* isolectins E4 and L4 to the microvilli and microvillous vesicles in the top area of the villi, was demonstrated by immuno-electron microscopy. No differences were observed concerning the distribution of the isolectins.

In the explants, cultured for five hours with the PHA isolectins, the enterocyte height and the villus length were decreased, and a lower villus/crypt ratio was calculated. At the ultrastructural level, the microvilli appeared to be shorter and irregularly positioned. After incubation with both PHA-E4 and PHA-L4 clusters of small vesicles, tied off from the microvilli, were seen in higher numbers when compared to control explants.

The activity of the brush border enzyme sucrase-isomaltase was decreased in the PHA-E4 incubated explants, whereas the enzyme activity did not change in the PHA-L4 incubated explants.

This present investigation clearly shows that explants of pig jejunal mucosa in organ culture are suitable to study the pathological effects of lectins on the small intestinal mucosa.

Introduction

In recent surveys the ubiquitous presence of lectins in plants (e.g. seeds of legumes like pulses and beans) eaten by man and his farm animals are described[5,11,12,17].

Essentially, the biological activity of lectins can be abolished by proper heat treatment, although heat processing is not always very effective[19]. Since most lectins are resistant to proteolytic breakdown in the gastrointestinal tract, they can reach the small intestine in a biological active form and bind to the glycoconjugates of the mucus and glycocalyx[1,20]. Pusztai *et al.*[18,20] suggest that many of these lectins may account for the morphological and functional changes in the epithelium of the small intestine, leading to a reduction of the efficiency of nutrient utilization. In spite of several studies the pathogenesis of lectin induced changes in the intestinal tract has not fully been elucidated[7,13,23]. In order to study the effects of lectins on the small intestinal mucosa, without any interference from the luminal contents and the intestinal micro flora[1,28] *in vitro* studies are most promising in that respect. Moreover, the experimental conditions of the mucosal explants can be controlled more precisely in comparison to the *in vivo* situation[25] and therefore may allow to single out the direct effect of any given deleterious agent on the mucosa. The fact that only small amounts of deleterious material are needed in an *in vitro* system is also in favor of an in vitro approach.
Therefore, explants of pig jejunal mucosa in organ culture, being closely related to the situation *in vivo*, were chosen to study the interaction between *P. vulgaris* isolectins E4 (4 erythro-agglutinating subunits) and L4 (4 leuco-agglutinating subunits) and the jejunal mucosa *in vitro*. We have investigated the binding of PHA-isolectins E4 and L4 to the explants and the subsequent lectin induced effects on explant morphology and brush border enzyme activity.

Materials and methods

Approval for the experiments was given by the ethical and experimental committee of TNO-CIVO, Zeist, The Netherlands.

Four 7 weeks old, male SPF-pigs (CDI, Lelystad, The Netherlands) were premedicated with Stressnil^{R} and atropine and anaesthetized with fluothane and nitrous oxide in oxygen. The abdomen was opened and four jejunal segments 20 cm in length were taken 3 meters proximal to the ileocaecal ligament. Explants were prepared according to Danielsen *et al*[4]. The segments were cut open longitudinally at the mesenterial attachment and rinsed in Trowell's T8 medium at 21° C[26]. With a scalpel the mucosa was separated from the submucosa and small explants ($\pm$ 9 mm^2) were cut from the mucosa. The explants were cultured according to the method described by Browning and Trier[2]. Briefly, the

culture conditions are as follows. Explants were placed on triangular stainless steel grids, the villus side facing upwards (6 explants per grid), in sterile plastic organ-culture dishes (Falcon^{R} 3037). To the central well of these dishes 0.9 ml of Trowell's T8 medium was added, until a thin layer was drawn over the villous surface of the explants by capillary action. In the isolectin-experiments, 50 μg of the PHA-E4 and PHA-L4 isolectins (E-Y Laboratories Inc., San Mateo, California, U.S.A.) were added per ml of Trowell's T8 medium respectively. The outer ring of the dish contained 3 ml of bidistilled water. The dishes were placed in an airtight container, gassed with a mixture of 95% O_2 and CO_2 for 15 minutes) and placed in an incubator (37° C)[2].
Explants cultured for 5 hours in Trowell's T8 medium and explants cultured for 5 hours in Trowell's T8 medium in the presence of 50 μg per ml of the *P. vulgaris* isolectins E4 and L4 respectively, were processed for histomorphometric, electronmicroscopic and biochemical examinations.

Tissue processing for morphometric analysis

For morphometric analysis one explant of each dish was fixed in 0.1 M phosphate-buffered 4% formalin, pH 7.3, dehydrated and embedded in paraffin. Serial sections (5 μm) were cut and stained with haematoxylin and eosin (HE). Using these sections, 10 well orientated villi and crypts were measured by means of a TEA Image Manager system (DIFA, Breda, The Netherlands). The height of the villus was represented by the distance from the crypt opening to the tip of the villus, and the crypt depth from the base of the crypt to the level of the crypt opening. The villus/crypt ratio was calculated to indicate the extent of morphological abnormality. The same sections were used to measure the enterocyte height from at least 25 epithelial cells with basal-centrally situated nuclei distributed randomly over the villi. Crypt enterocytes as well as enterocytes in the extrusion zone were excluded from these measurements.

Tissue processing for scanning electron microscopy

One explant per dish was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.35; 440 mOsm) for 24 hours at 4°C, and then rinsed in 0.1 M cacodylate buffer (pH 7.35). Subsequently, the fixed explants were mounted on stubs with the villus side upward, treated according to the OTOTO method (alternating osmium tetroxide and thiocarbohydrazide immersion)[15], dehydrated through graded concentrations of ethanol, transferred to amylacetate, critical point dried with CO_2, and mounted on aluminum stubs with silver paint. Specimens were examined using a Cambridge Camscan scanning electron microscope at 10 kV.

Tissue processing for transmission electron microscopy

One explant per dish was fixed in 0.1 M cacodylate buffered 2.5 % glutaraldehyde (pH 7.35, 440 mOsm) during 24 h at 4°C, and subsequently rinsed in 0.1 M cacodylate buffer (pH 7.35). After postfixation with 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.35) for 16 h at 4°C, the explants were dehydrated in graded water-aceton mixtures and embedded in an Epon Araldite mixture. Semithin sections were stained with toluidine blue. Ultrathin sections were stained with uranylmagnesium acetate and leadcitrate and examined with a Philips EM410LS electron microscope at 60 kV.

Tissue processing for immuno-electron microscopy

One explant per dish was fixed in 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.35) for 7 days at 4° C and subsequently embedded in Lowicryl (polymerised at 20° C with 360 nm wavelength UV light). Ultrathin sections were cut with a glassknife on a Reichert Ultracult E ultramicrotome and collected on uncoated 150 mesh copper grids. The primary antibodies used in this study were respectively rabbit anti-PHA-E4 and rabbit anti-PHA-L4 (E-Y Laboratories Inc., San Mateo, California U.S.A.). Section labeling was performed by floating grids, sections down, on droplets placed on a strip of parafilm. All incubations were carried out at room temperature. The immunolabeling procedure is outlined as follows. Sections were incubated and pretreated on 20 μl droplets of 1% bovine serum albumine (BSA) (Organon, Oss, The Netherlands) in 0.1 M phosphate-buffered saline, pH 7.4 (1% BSA/PBS) for 15 minutes. Excess buffer was removed with pieces of filtering paper before the grids were incubated on 20 μl droplets consisting of antibody diluted in 1% BSA/PBS for 30 minutes. The final dilution of both primary antibodies was 1:160 with 1% BSA/PBS. After incubation the grids were washed 3 times for 5 minutes by floating, sections down, on fresh 20 μl droplets of 1% BSA/PBS. Excess buffer was removed prior to incubation for 30 minutes with colloidal gold labeled secondary antibody. In this study goat-anti rabbit IgG conjugated to 15 nm colloidal gold (EM grade) (Janssen Pharmaceutica, Beerse, Belgium) was used in a final dilution of 1:20. Before counterstaining with uranyl magnesium acetate and lead citrate, the grids were washed 3 times for 5 minutes on droplets of bidistilled water. Grids were air-dried and examined with a Philips EM410LS electron microscope at 60 kV. Control sections were treated as above, omitting the primary antibody-incubation step[22].

Sucrase-isomaltase enzyme activity assay

Three explants from each dish were frozen (liquid nitrogen) and stored at -70°C before analysis. The explants were pooled (3 explants for each time point

or PHA concentration) in 2 ml of 2 mM TRIS-50 mM mannitolbuffer (pH 7.1). From here all procedures were performed at 4°C, unless mentioned otherwise. The explants were sonified twice for 10 s in 1 ml of bidistilled water. After sonification an aliquot of the suspension was used for protein determination[14]. Sucrase-isomaltase (EC 3.2.1.48) activity was determined according to Messer and Dahlqvist[16], using sucrose (Serva, Heidelberg, Germany) as substrate. The specific enzyme activity was calculated as U/g of protein and expressed as the mean relative specific enzyme activity ± SEM.

Statistical analysis

The statistical significance of differences between means was analyzed using the paired student's t-test. In the tables the values are expressed as percentage of the corresponding controls.

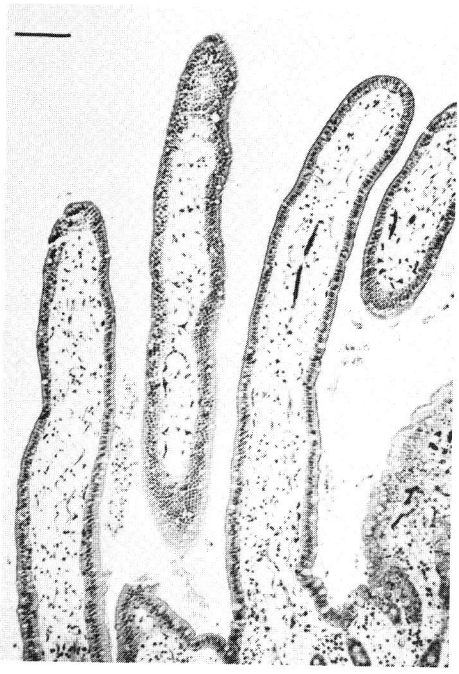
Results

Morphologic characteristics of pig jejunal explants

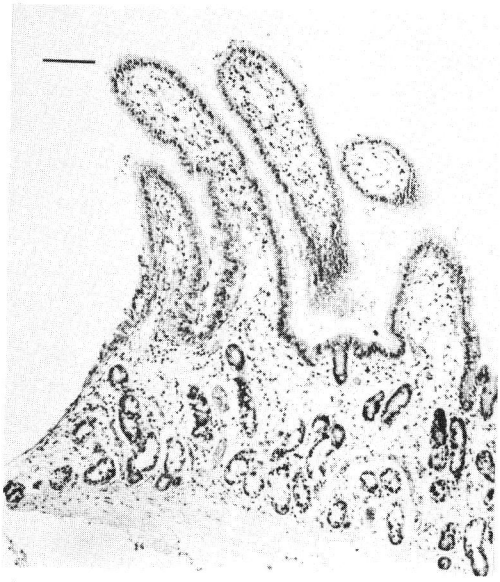
The histology of the control explants, PHA-E4 and PHA-L4 cultured explants are depicted in the Figures 1a, b, c, respectively.

The results of the morphometric analysis of pig jejunal explants from PHA-E4 and PHA-L4 incubated and control explants are summarized in Table 1. The morphology of the control explants, cultured for 5 hours in Trowell's T-8 medium appeared normal by light microscopy. In explants cultured in the presence of the PHA-isolectins E4 and L4, a remarkable decrease of the villus/crypt ratio was observed. Incubation of the explants with the isolectins caused a decrease of 30.1% for PHA-E4 and 36.7% for PHA-L4. As shown in Table 1 the PHA-induced decrease in the villus/crypt ratio of the explants was due to a change of the villus length only, whereas the PHA-L4 induced decrease was accomplished by both a decrease of the villus length and an increase of the crypt depth. After incubation of the explants for 5 hours with PHA-isolectin E4 the mean enterocyte height decreased with 18.4 %, whereas incubation with PHA-isolectin L4 resulted in a decrease of 10.6 %. Each of these decreases were statistically significant ($p < 0.01$).

Figure 1. Effects of PHA-isolectins on pig mucosal explants in organ culture. A. Control explant cultured for 5 hours in Trowell's T-8 medium. B. Explant cultured in Trowell's T-8 supplemented with 50 μg of PHA-E4/ml, and C, with 50 μg of PHA-L4/ml. The depth of the crypts remains unchanged. (A. Bar = 10 mm; B, C. Bar = 11 mm).



1A



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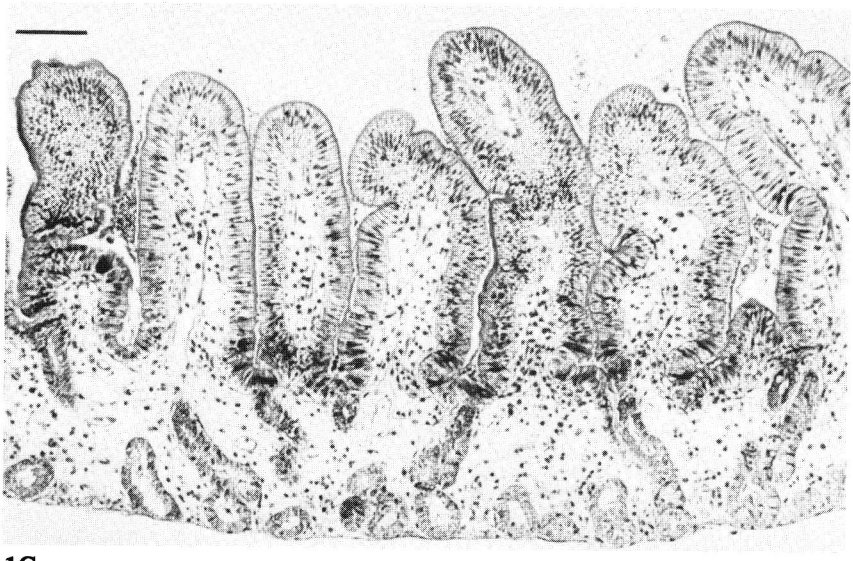
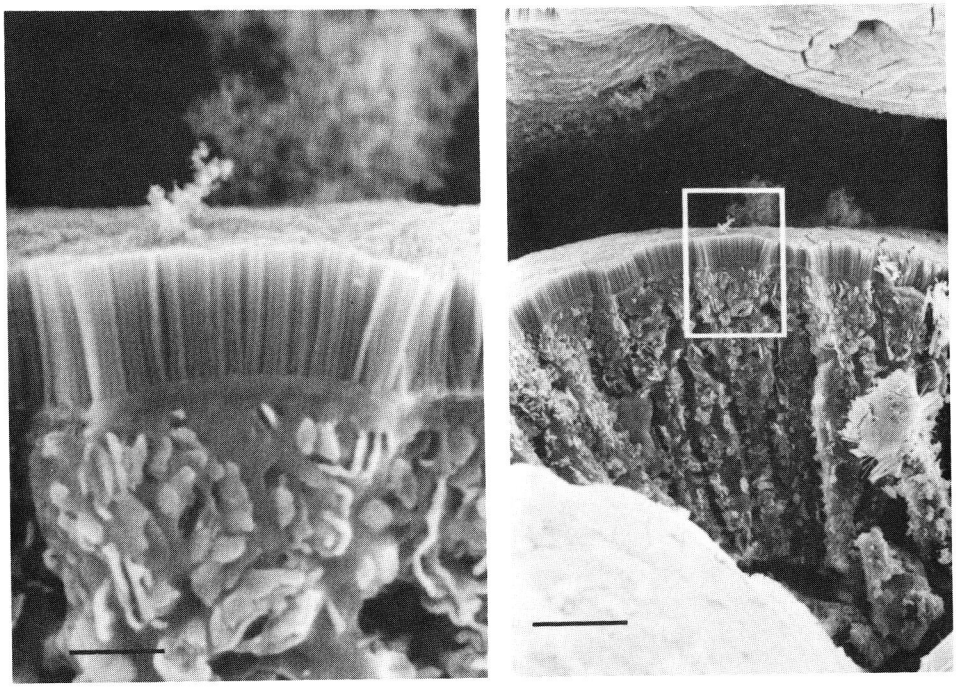


1C

Table 1. Effects of *Phaseolus vulgaris* isolectins on the length of villi, the depth of crypts, the villus/crypt ratio, and the height of enterocytes in pig jejunal explants.

	Villus	Crypt	Villus/crypt ratio	N	Enterocytes	N
Controls	100.0 ± 2.9	100.0 ± 2.5	100.0 ± 9.9	70	100.0 ± 1.3	278
PHA-E4	70.5 ± 5.5*	101.0 ± 6.8	69.9 ± 5.9*	70	81.6 ± 1.6*	278
Controls	100.0 ± 2.7	100.0 ± 2.9	100.0 ± 5.0	30	100.0 ± 2.5	115
PHA-L4	74.4 ± 1.7*	114.6 ± 5.4	63.3 ± 2.5*	30	89.4 ± 1.4*	115

Values are given as mean relative length, depth, ratio, and height ± SEM. *P < 0.01 compared with controls



2A 2B

Figure 2. Scanning electron micrograph of a pig mucosal explant cultured in Trowell's T-8 medium (A. Bar = 5 μm). The microvilli are regularly shaped; some mucus is expelled on the surface (B. Bar= 25 μm).

Ultrastructural changes in the explants after incubation with PHA-isolectins

The scanning electron-microscopic studies revealed that culturing explants for 5 hours in Trowell's T8 medium did not influence the morphology of villi and microvilli (Figures 2A, B). However, as demonstrated in Figures 3 A-D exposure of the explants to 50 μg/ml of PHA-E4 and PHA-L4, respectively, resulted in changes in the morphology of the villi and microvilli. In the PHA-E4 cultured explants a pock pitted surface of the villi and fusion of microvilli was seen (Figures 3A, B). The villi of the PHA-L4 cultured explants were covered with dots of material (Figures 3C,D), which on cross section seemed to be derived from the microvilli.

The transmission electron microscopic study of control explants, cultured in Trowell's T-8 medium, revealed microvilli which were even in length and regularly positioned (Figures 4A, 5A). The core of the microvilli, consisting of bundled actin filaments, was clearly seen to protrude into the cytoplasm. No vesicles appeared to be tied off from the microvillar tops. However, explants cultured in the presence of the PHA-E4 (Figures 4B, 5B) and PHA-L4 (Figure 4C) isolectins respectively, showed a clear shortening of the microvilli of epithelial cells of the upper one third of the villi. The microvilli were irregularly positioned and fusion frequently occurred. In addition, vast numbers of microvillous vesicles had been tied off and areas could be observed where a complete loss of microvilli had taken place. The described changes however, seem less abundant in the PHA-L4 treated explants when compared to the PHA-E4 treated ones.

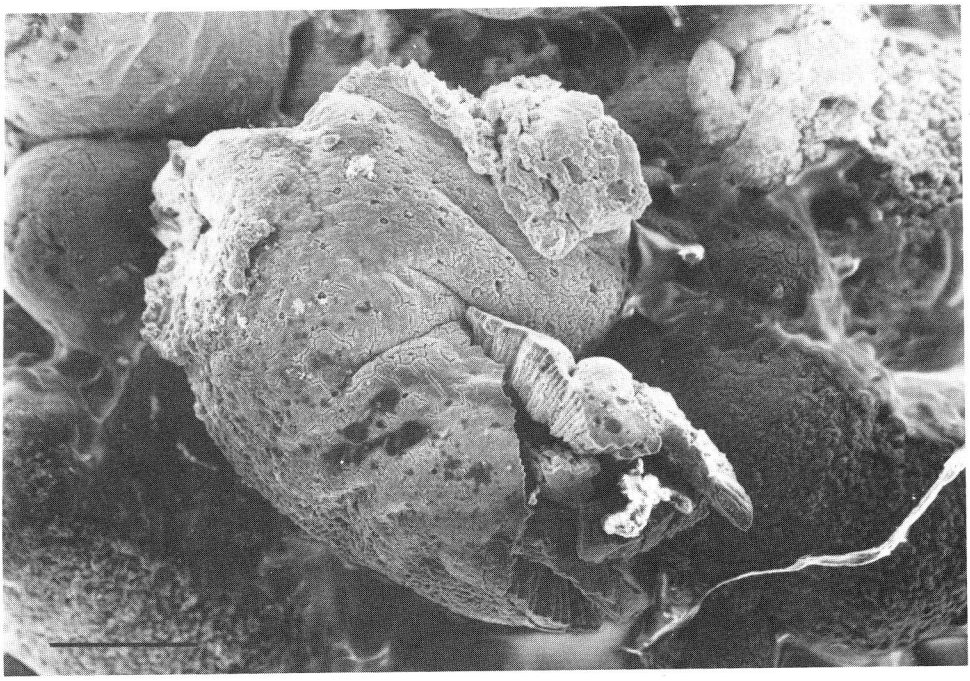
Binding of PHA-isolectins E4 and L4 to pig jejunal explants

As visualized by immuno electron microscopy, PHA-E4 (Figure 6A) as well as PHA-L4 (Figure 6B) bind to the microvilli of the cultured explants. No differences could be detected with respect to the distribution of gold particles. The gold particles were located almost exclusively over the microvilli and over the tied off microvillous vesicles. Staining was more evident in the top one third area of the villi, however gold particles were also detected over the microvilli along the length of the villi and over the mucous contents of goblets cells. No gold particles could be detected in control explants.

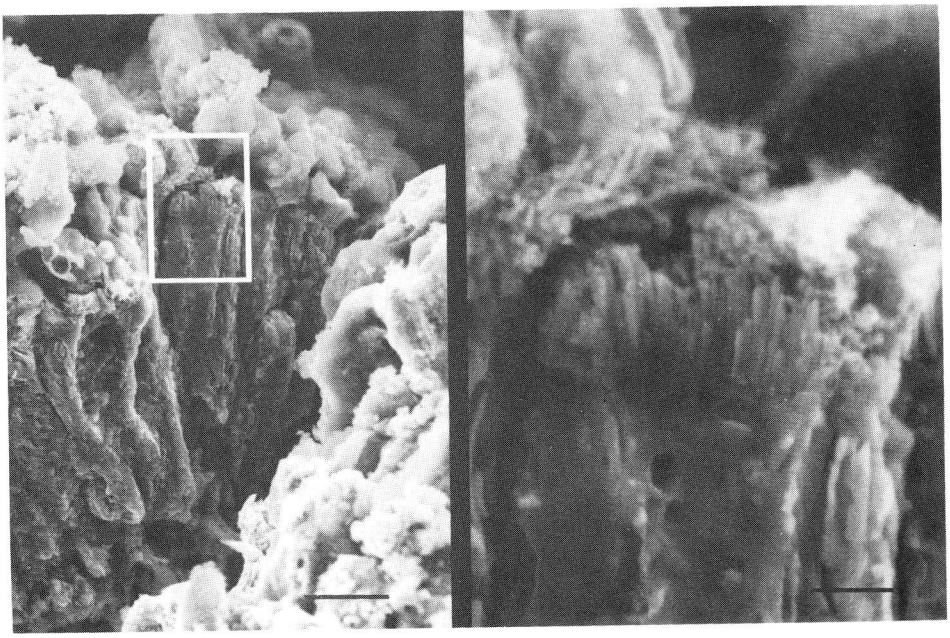
Effect of PHA-isolectins on the brush border enzyme activity

The effect of PHA-isolectins E4 and L4 on the specific activity of the brush border enzyme sucrase-isomaltase in pig jejunal explants is demonstrated in Table 2.

Incubation of the explants in the presence of 50 μg of PHA-E4/ml resulted in a significant decrease of the relative specific activity as compared to control explants cultured in Trowell's T-8 medium (Table 2). PHA-L4 at a concentration



3A



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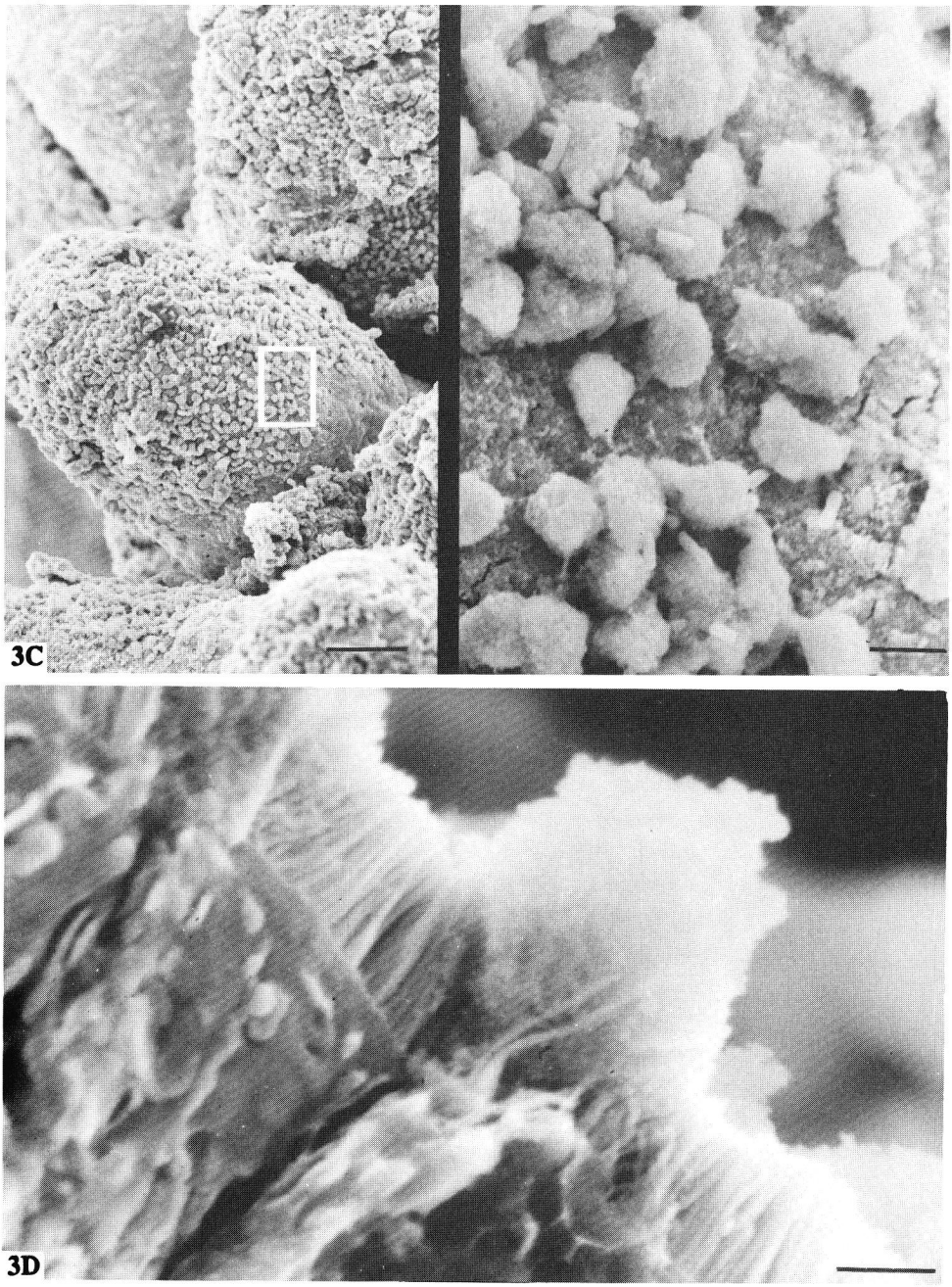
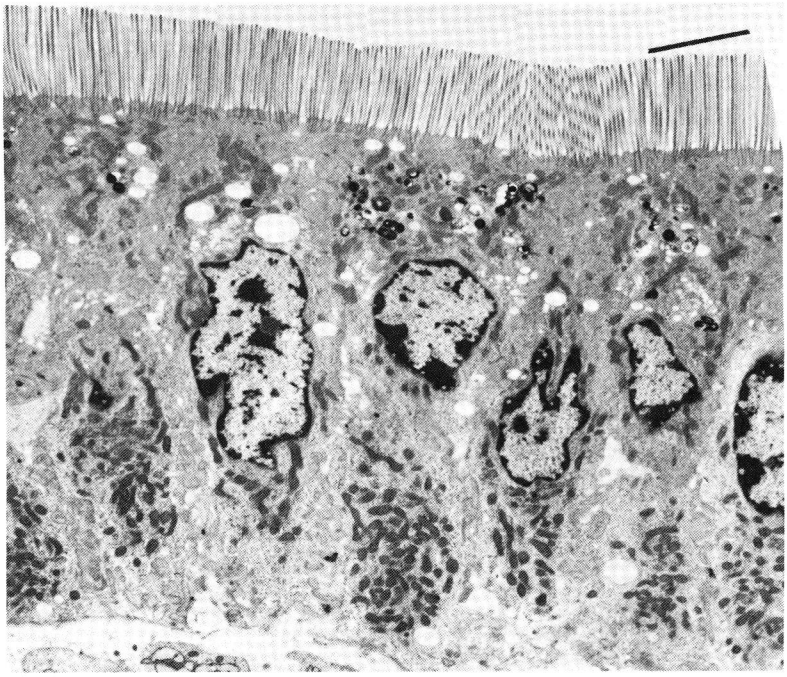


Figure 3. The surface of mucosal explants after exposure to 50 μg of PHA-E4/ml (A,B) or 50 μg of PHA-L4/ml (C,D) of Trowell's T-8 medium. A. Note the pock-pitted surface of the villi, and irregularity of the microvilli (B). C, D. Note the dots on the surface of the microvilli. (A, C. Bar = 50 μm; B, D. Bar = 1.5 μm).



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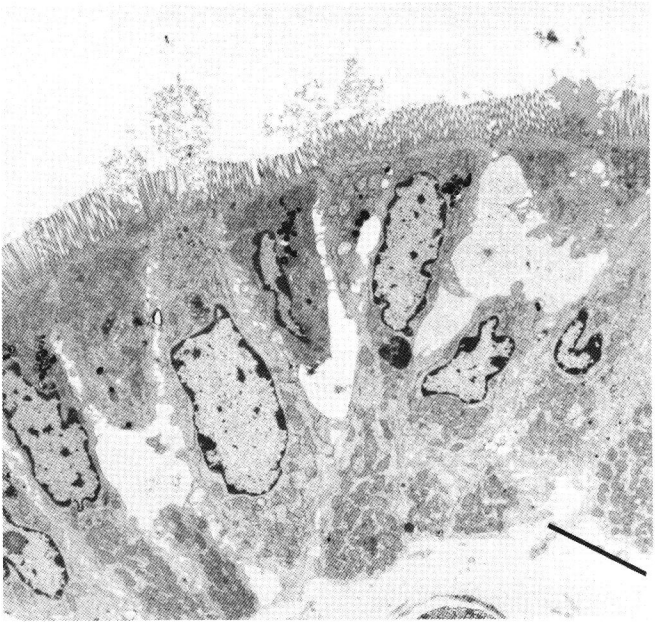

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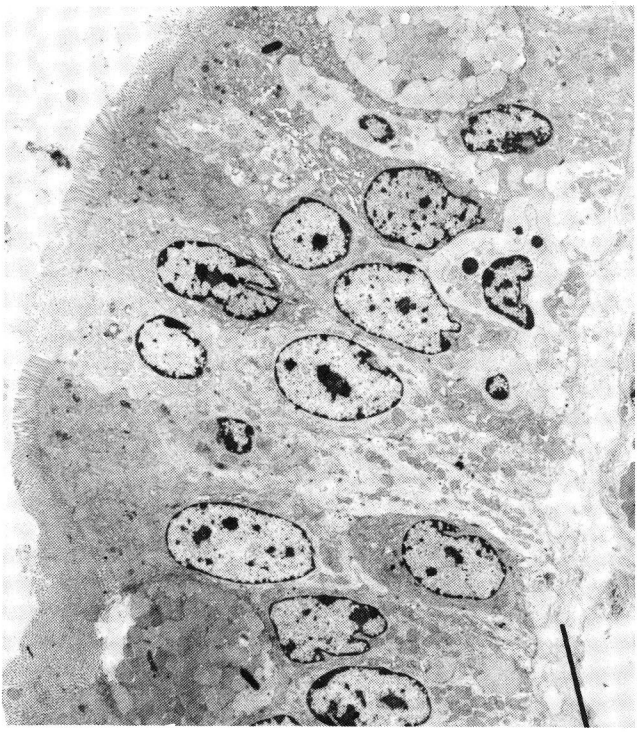


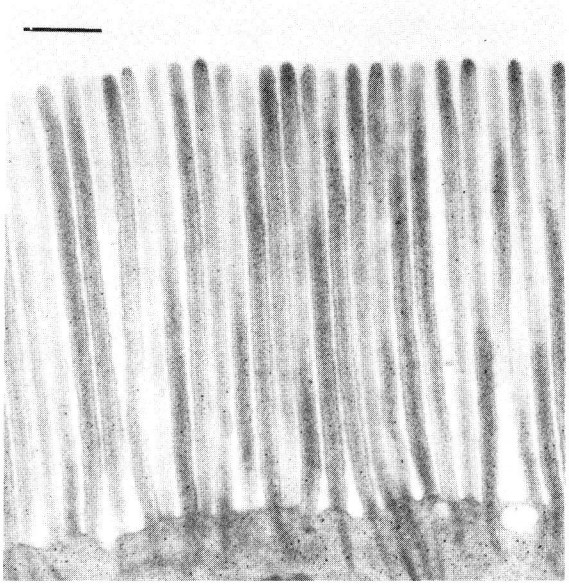
4C

Figure 4. The absorptive epithelium from pig mucosal explants cultured for 5 hours in Trowell's T-8 medium (A), in the presence of 50 μg of PHA-E4/ml (B) and PHA-L4/ml (C) of Trowell's T-8 medium. A. The epithelium appears normal. B. Note the masses of microvillous vesicles. C. Same but less abundant. (A, B, C. Bar = 380 μm).

Table 2. The effects of *Phaseolus vulgaris* isolectins on the specific activity of the brush border enzyme sucrase-isomaltase in pig jejunal explants.

	Activity of sucrase-isomaltase	N
Controls	100.0 ± 5.2	56
PHA-E4	82.9 ± 11.4[a]	56
Controls	100.0 ± 2.2	24
PHA-L4	98.7 ± 9.9	24

Activities were measured in units per gram of protein. Values are expressed as the mean relative specicific activity ± SEM. [a]$P < 0.01$ compared with controls.



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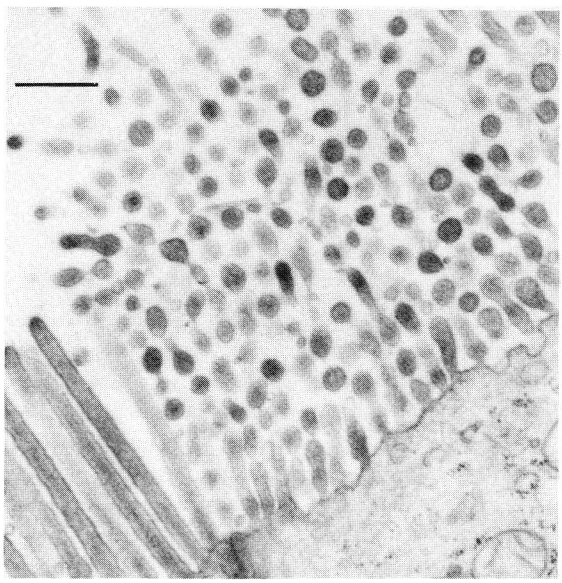
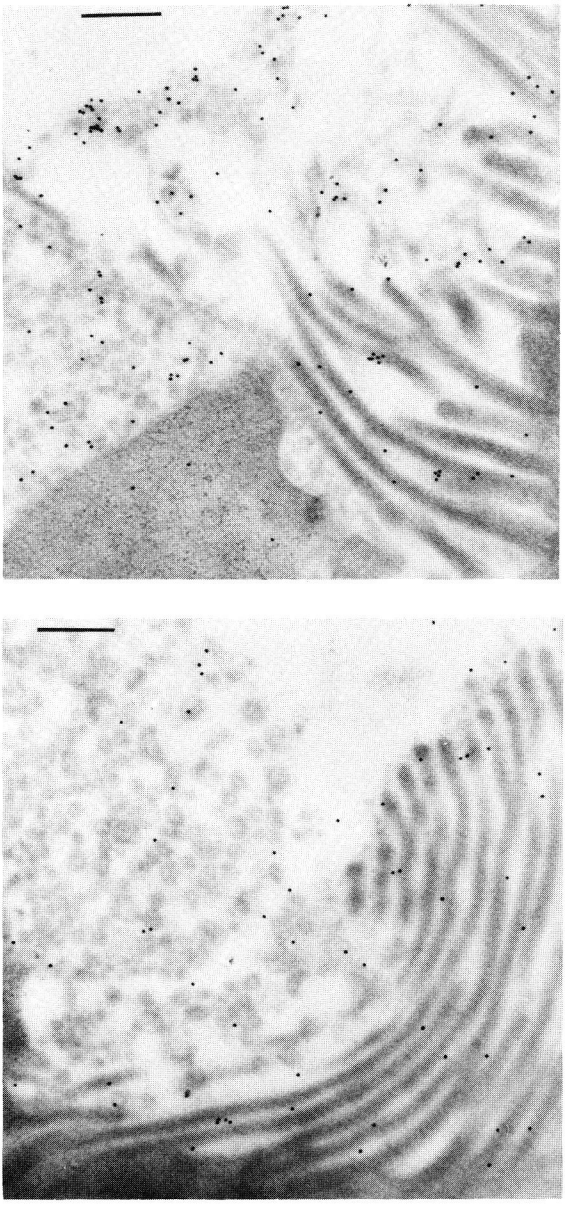


5B

Figure 5. The microvilli of the absorptive epithelium from pig mucosal explants cultured for 5 hours in Trowell's T-8 medium (A), in the presence of 50 μg of PHA-E4/ml (B) and 50 μg of PHA-L4/ml (C) of Trowell's T-8 medium. A. The microvilli are even in length, and regularly positioned. The fibrillary roots clearly protrude into the cytoplasm. B. The microvilli are irregularly positioned and shortened, microvillous vesicles are tied off. The microvilli are fragmentary. (A, B. Bar = 32 μm).



6A

6B

Figure 6. Immunogold localization of PHA-E4 (A) and PHA-L4 (B) in the brush border and apical cytoplasm of an absorptive enterocyte in a pig mucosal explant. Gold particles are almost exclusively located over the microvilli in the top area of the villi and tied off vesicles. No differences could be detected between the isolectins with respect to the localization of their binding. (A, B. Bar = 50 μm).

of 50 μg/ml did not influence the relative specific activity of sucrase-isomaltase.

Discussion

The objective of the present study was to investigate whether organ culture from pig small intestinal mucosal explants represents a suitable model in studying deleterious effects of PHA-isolectins E4 and L4 on enterocyte morphology and function. And if so this model may be used in the elucidation of the mechanisms of damage to small intestinal mucosal structure.

When small intestinal mucosal explants are cultured upto 5 hours in Trowell's T-8 medium, only minor changes occur in the histological as well as ultrastructural architecture (Figures 1A, 2, 4A, 5A). Also other investigators report, that based on light and electron microscopic criteria, the mucosal morphology of pig small intestinal explants is well preserved during organ culture.[3] The minor changes in the mucosal morphology, which take place during culture, are presumably due to the reflection of the *in vitro* environment.[6]

Exposure of the pig jejunal explants for 5 hours to PHA-E4 and PHA-L4 provokes numerous morphological changes (Table 1, Figures 1B,C, 3, 4B,C, 5B). These changes enclose a significant decrease in mean enterocyte height, a decrease in villus/crypt ratio due to a decreased villus length and increased crypt depth in PHA-L4 treated explants, and the presence of shortened and irregularly positioned microvilli with vast numbers of tied off microvillous vesicles. Furthermore, in the PHA-E4 treated explants the specific activity of the brush border membrane enzym sucrase-isomaltase was decreased significantly (Table 2). No significant difference could be detected in the PHA-L4 treated explants.

By electron microscope immunocytochemistry, immunogold localization of PHA-E4 and PHA-L4 was demonstrated over the glycocalyx of the intestinal microvilli, microvillous vesicles in the top one third area of the villi (Figure 6), and less over the microvilli of enterocytes in the lower parts of the villi.[9] No differences were observed between the distribution of the isolectins on the cells, which is in agreement with the findings of King *et al.*[10] in rats fed raw kidney bean protein.

Extensive alterations of small intestinal enterocytes, decrease in villus length, irregularity of microvilli, clusters of vesicles associated with the brush border, and complete loss of microvilli, are described in rats after intraluminal administration of Concanavalin A or Wheat germ agglutinin.[13] When rats (conventional as well as germ free) were fed purified PHA, and adherence of PHA to the brush border membrane was obvious, none of these morphological changes of the small intestinal mucosa could be observed.[1] However, rats fed a diet containing crude Phaseolus vulgaris beans display an extensive disruption and abnormal structure of microvilli in the small intestine.[7,27]

Our findings show that exposure of pig mucosal explants for 5 hours to

PHA-E4 or PHA-L4 results in binding of the isolectins to the intestinal epithelium, brush border membrane alterations, diminished length of villi and in case of PHA-E4 incubated explants a decreased activity of sucrase-isomaltase.

Several mechanisms, such as the Globular(G):Filamentous(F)-actin ratio, the cell actin content, the cytoskeletal protein turnover and/or the pool size of actin binding proteins, might be involved in the regulation of the the intestinal brush border villus length.[24] In differentiated human colon carcinoma Caco-2 cells exposed to soybean agglutinin we have demonstrated that the shortening of the microvillus length is accomplished only by a rapid shift in the G:F-actin ratio.[4] After binding of the PHA-isolectins to the brush border membrane of the enterocytes, changes in the actin cytoskeleton of the cell probably plays a role in the pathogenesis of microvillous abnormalities. After exposure of rat intestinal epithelium to intraluminal dietary lectins clusters of vesicles associated with the brush border can be found.[8,13] Also in organ culture vesiculation of microvilli leading to increased numbers of vesicles nearby the brush borders of PHA-isolectins incubated explants, were observed (Figure 5). The presence of these vesicles might reflect an increased turnover of microvillous membrane.

The loss of brush border membranes interferes at once with the activity of brush border associated enzymes. Our results demonstrate a correlation between the amount of microvillous vesicles which have been tied off and the decrease in activity of sucrase-isomaltase (Table 2).

This similarity between the lectin-induced lesions *in vivo*[8,13] and *in vitro* responses of pig mucosal explants clearly demonstrate that organ culture of pig mucosal explants represents a suitable model for further *in vitro* studies on the pathogenesis of the lectin induced lesions in the pig small intestine.

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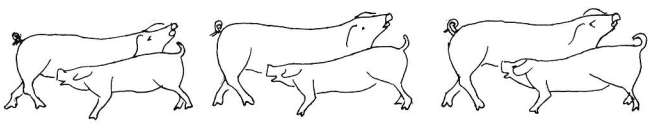
CHAPTER VI

A SMALL INTESTINAL BIOPSY TECHNIQUE IN CANNULATED PIGLETS



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After: Journal of Animal Physiology and Animal Nutrition 1988;**60**:123-7.
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Summary

In order to create the possibility of studying the small intestinal mucosa on successive occasions during life in the same animal, a biopsy technique has been developed in cannulated piglets. A cannula has been placed in the jejunum through which the mucosal biopsies were taken with a blind-biopsy tube.

Zusammenfassung

Eine Dünndarm-Schleimhaut-Biopsie Technik im fistulierter Schwein.
Um die Möglichkeit zu schaffen, die Dünndarm Schleimhaut bei aufeinanderfolgenden Gelegenheiten während des Lebens eines Schweines zu studieren, wurde eine Biopsie-Technik entwickelt in fistulierten Schweinen. Eine Fistula wurde gestaltet im Jejunum vom Schwein, durch welche mit einer blinden Biopsie Tube Schleimhaut-Biopsien genommen wurden .

Introduction

As far as we know, up to now porcine small intestinal mucosal samples for pathological studies have been obtained after killing the animal[1,5,6]. There are few reports based on examinations of the small intestinal mucosa in experimental piglets during life[2,7], and in these studies the small intestinal specimens were obtained during laparotomy after which the animals were killed.

In this article a small intestinal biopsy technique in cannulated piglets, which allows the sampling of mucosal specimens during life on successive occasions in the same animal, is presented.

Materials and methods

The piglets were individually housed in raised pens and fed a standard diet. Two 8-week-old, clinically healthy, Great Yorkshire x Dutch Landrace conventional piglets of 12 kg body weight were, after 24 hours of fasting, premedicated and anaesthetized through inhalation anaesthesia according to Lumb and Jones[4]. Lidocaine with epinephrine (8cc) was administered in the area of the incisions subcutaneously and intramuscularly. The operation area was shaved and disinfected with a general disinfectant.

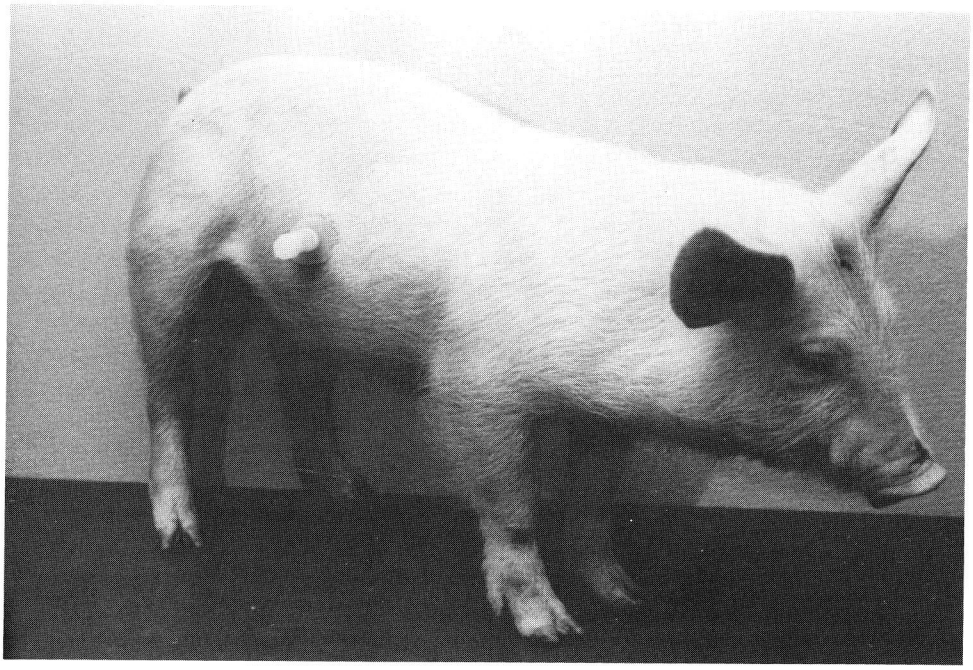

Figure 1. Pig with the jejunal cannula, 21 days post operative.

An incision (about 20 cm in length) was made in the right abdominal wall, ventrocaudal to the last rib, starting 20 cm below the transverse process of the first lumbar vertebra, opening the abdominal cavity. About 80 cm caudal to the Ligament of Treitz, a cannula was placed in the intestinal lumen at the antimesenteric side of the jejunal wall, and led out through the abdominal wall, and the incision was closed[3] (Figure 1).
The cannula was made of Silastic Medical Adhesive Silicone Type A (Dow Corning Corporation Medical Products, Midland, Michigan, U.S.A. 48640) and manufactured according to figure 2.

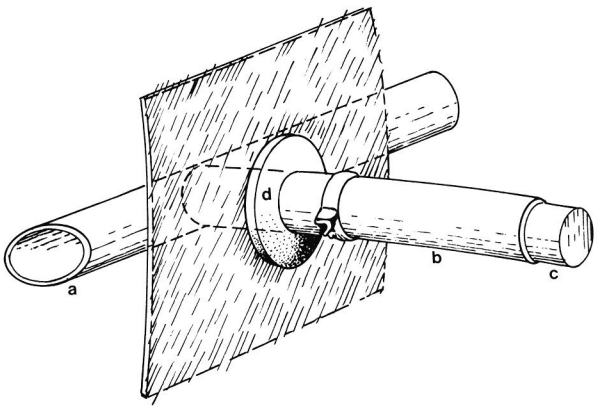

Figure 2. Jejunal cannula made of Silastic medical plastic.
a. flanged end, which is inserted in the jejunum.
b. barrel which is led out through the skin.
c. plug for closing the cannula.
d. polyester ring for keeping the cannula in its place.

The biopsy tube used was a slightly modified Laméris blind-biopsy tube for adults, nr. 546 (Laméris, Medical Equipment, Utrecht, The Netherlands) figure 3, which in previous tests in calves proved to give suitable results (not published). The biopsy tube has at its end a metal cylinder containing a 2.5 mm hole in its wall. The biopsy tube was inserted in the jejunum through the cannula and pushed distally approximately 50 cm, where the biopsies were taken. Intestinal tissue was aspirated through the hole into the cylinder, cut off by rotation of the cutter, and collected.

In each pig the biopsies were taken on 12 different occasions, with 1 or 2 days interval, and fixed in 4% buffered formalin solution. The biopsies were examined stereomicroscopically for the three-dimensional structure of the villi, and the presence of artefacts (e.g., redness).
Perpendicular dissections of the fixed specimens were embedded in paraffin and

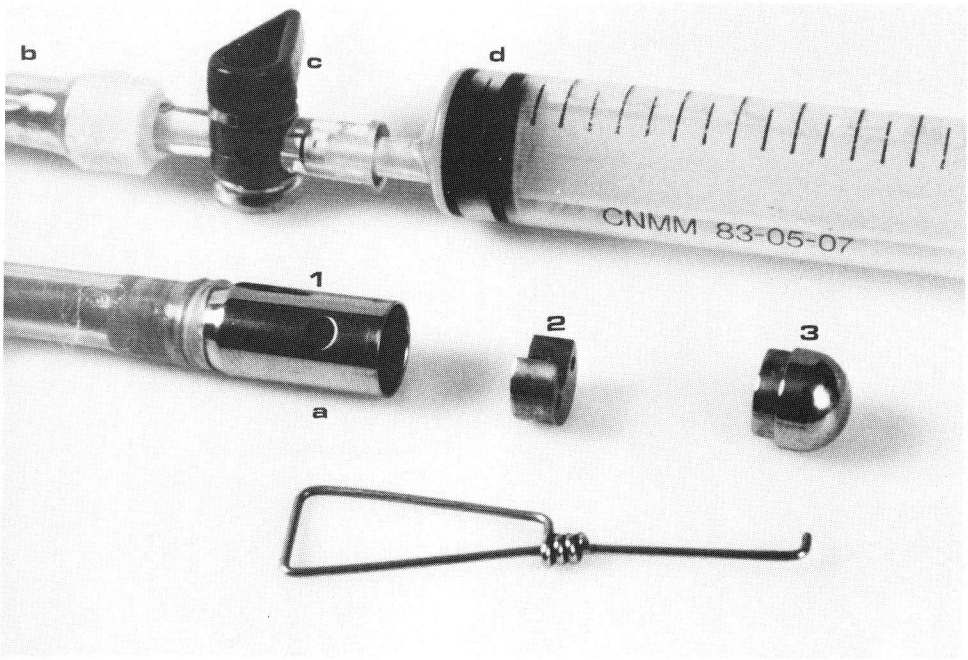

Figure 3. Lameris blind-biopsy-tube for adults no. 546.
a. iron cylinder on the top of the tube with hole (1), cutter (2) and closing cap (3)
b. tube.
c. valve
d. 50 cc syringe for suction.

5 μm sections were stained with hematoxylin and eosin (H.E.), and were histologically evaluated for depth of the biopsy and structure of the mucosa.

Results and discussion

The size of the biopsies ranged from 4 mm to 8 mm in length and approximately 2.5 mm in width. Stereomicroscopic inspection showed that the villus pattern had been well preserved (figure 4) and that the muscularis mucosae was included. In only some of the biopsies a slight redness, probably due to suction, was seen.
Histologically, in most of the sections the mucosa was intact and usually included the muscularis mucosae (figure 5).
In man, the inclusion of the muscularis mucosae in the mucosal biopsy appeared to be important for the preservation of the villous structure[8].

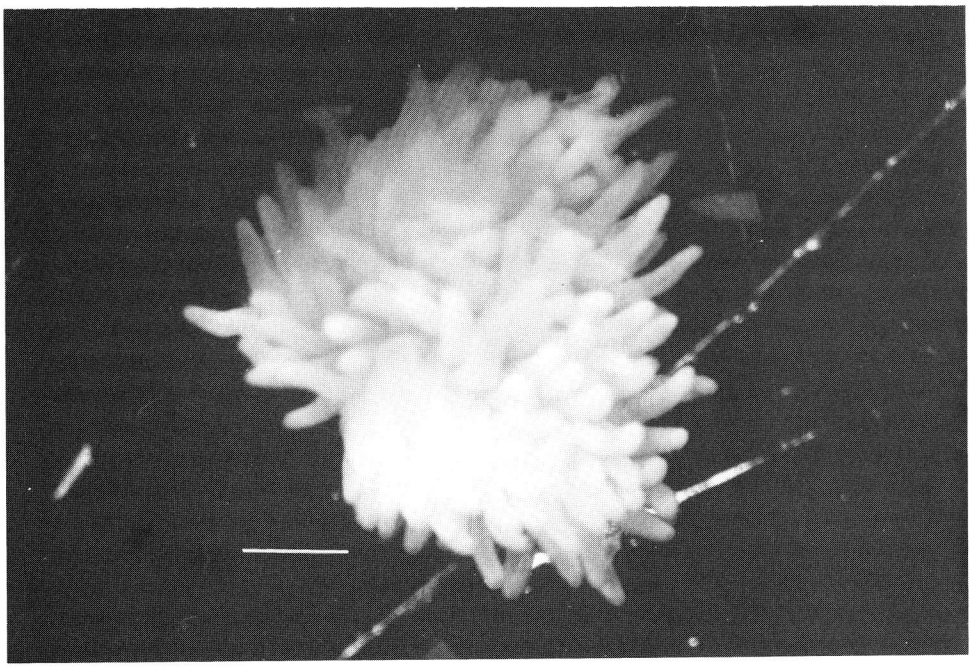

Figure 4. Stereomicroscopical picture of a biopsy (bar is 0.1 mm).

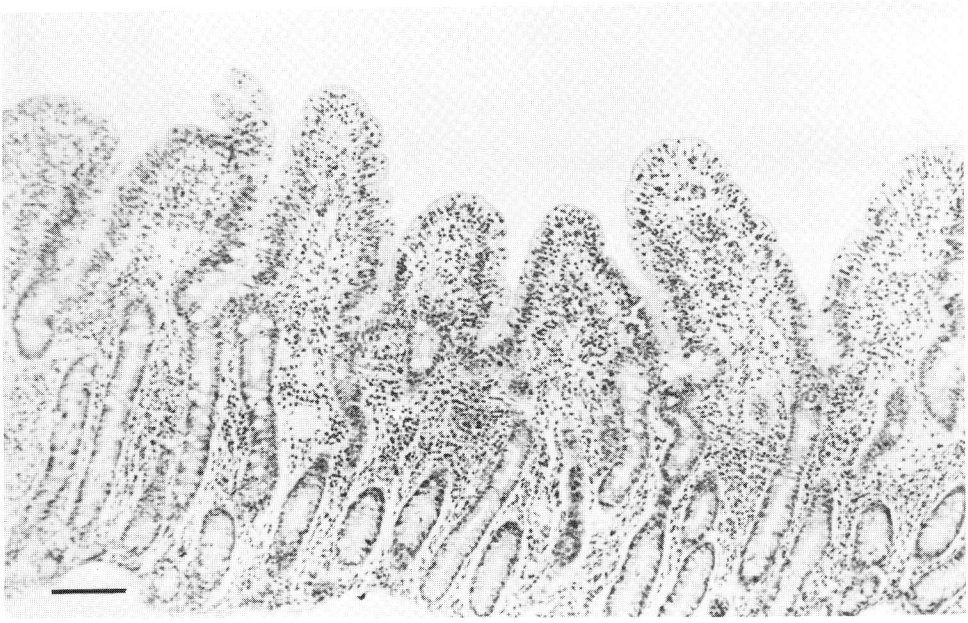

Figure 5. Histological picture of a section of a biopsy. H.E. (bar is 0.1 mm).

It may be concluded that using this biopsy technique, mucosal biopsies of sufficient morphological quality can be obtained. Furthermore, it proved possible to collect several mucosal samples of intestinal mucosa from the same animal on consecutive occasions. The implanted cannula and the technique of sampling does not seem to have influenced the health or growth of the animals.

This small intestinal biopsy technique in cannulated piglets allows the study of the interaction between administered noxious agents and the mucosal structure at consecutive times in the same animal. Moreover, with this method, fewer animals are needed in an experimental study, since an experimental animal can also serve as its own control, before and after the experimental treatment.

Acknowledgements

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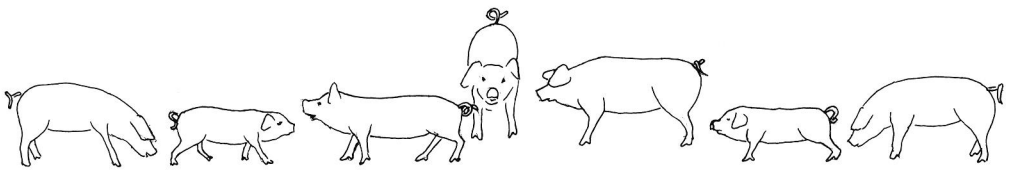
CHAPTER VII

DEVELOPMENT OF A SELF-EMPTYING BLIND POUCH (SEP) MODEL IN THE JEJUNUM OF PIGLETS



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After: Journal of Animal Physiology and Animal Nutrition, in press (printed with kind permission of the editor)

Summary

In order to investigate the effects of small amounts of enterotoxic substances in situ, a self-emptying blind pouch model has been made operationally in the jejunum of pigs. For this purpose piglets, 2 weeks of age were surgically provided with a one side closed self-emptying blind pouch (SEP) with intact blood vessel and nerve supply. At the blind end of the pouch a cannula was fitted. Through this cannula biopsies from the jejunal mucosa were taken with a blind-biopsy tube. Biopsies were taken from 10 days post operatively twice a week during six weeks, and studied morphologically as well as functionally. During this period the jejunal mucosa in the Self Emptying blind Pouch did not change with regard to its morphological as well as functional characteristics.

Introduction

Until now the effects of antinutritional factors were studied in intact animals[6,7,4], in cell cultures of differentiating human colon carcinoma Caco-2 cells[1,2], and in organ culture of pig small intestinal mucosa[5]. The cytotoxic effects of lectins have also been investigated using an isolated self-emptying jejunal loop in the rat[8]. On the analogy of this model we have developed a self-emptying blind pouch (SEP) with intact blood vessel and nerve supply, and a cannula at its blind end, in the jejunum of piglets.

The advantages of the self-emptying blind pouch model in piglets are multifold. This model allows study of antinutritional effects of lectins in situ. Purified lectins, which are very expensive, are only needed in rather small amounts, and can be administered through the cannula, thus ruling out the influence of the stomach contents, in a well defined part of the small intestine. By taking samples of mucosal tissue through the cannula it is possible to analyse the effects of a single dose of lectins on successive occasions. Fewer animals are needed to perform the experiments, because preceeding and following the test period the animal functions as its own control.

In this paper we describe the development of a self-emptying blind pouch in the jejunum of the pig and characterize the mucosa in the pouch morphologically as well as functionally.

To determine the possible effects on the structure of the mucosa, caused by the presence of the pouch with its cannula, the morphology of the mucosa in the pouch was investigated during six weeks post operatively by light and electron microscopy. The enzyme activities of sucrase-isomaltase and aminopeptidase were determined to establish the impact of the pouch on the functional state of the mucosa.

Materials and methods

Animals

Two SPF piglets (CDI - Lelystad, The Netherlands), 6 weeks of age, were individually housed in overpressure stables in cages with flat walls in order to minimize the risk of dislodging the cannula. They were fed a diet of which the basic nutrient composition may be found in Table 1. Water was accessible ad libitum. The SPF status means that animals were free of enterotoxigenic *Escherichia coli*, *Treponema hyodysenteriae*, enzootic pneumonia, transmissible gastroenteritis and PED viruses, but not free of rota viruses. Sows were not vaccinated.

Table 1. Composition (%) of the diet.

Ingredient	%	Ingredient	%
Barley	10.00	Sunflower oil	1.00
Processed corn	35.00	Cellulose	1.75
Fishmeal	5.50	$CaCO_3$	0.88
Skimmed milk powder	5.00	$CaHPO_4.2H_2O$	0.50
Whey powder delactosed	5.00	NaCl	0.30
Meat meal tankage	2.00	$NaHCO_3$	0.55
Corn gluten feed	3.00	$KHCO_3$	0.55
Potato protein	4.00	DL-methionine	0.07
Corn starch	19.67	L-lysine HCl	0.18
Cane molasses	4.00	L-tryptophan	0.05
VTM[1]	1,00		

[1] The vitamin and mineral premix supplied per kg diet:
Vitamin A 9000 IE, Vitamin D3 1800 IE, Vitamin E 40 mg, Vitamin K3 3 mg, Vitamin B2 5 mg, Vitamin B12 40 μg, nicotinic acid 30 mg, panthotenic acid 12 mg, choline chloride 150 mg, Vitamin C 50 mg, $CuSO_4.5H_2O$ 20 mg, $ZnSO_4.H_2O$ 200 mg, MnO_2 70 mg, $FeSO_4.7H_2O$ 400 mg, $CuSO_4.7H_2O$ 2.5 mg, $Na_2SeO_3.5H_2O$ 0.2 mg, KJ 0.5 mg

Establishment of the Self-Emptying blind Pouch

After 24 hours of fasting the animals were premedicated and anesthetized through inhalation anesthesia according to Lumb and Jones[10]. The right side was shaved and disinfected with a general disinfectant.

An incision (about 20 cm in length) was made in the right abdominal wall, ventrocaudal to the last rib, starting 20 cm below the transverse process of the first lumbar vertebra, and the abdominal cavity was opened. About 4.0 m cranial to the ileocaecal ligament the jejunum was bisected with a GIA (Autosuture, Zeist, The Netherlands), and the thus formed bowel sides were simultaneously closed with a double staggered staple line. The mesentery was split towards the origin of its bloodvessels. The proximal end of the jejunum was brought 50 cm caudally and anastomosis was performed side to side. Briefly, the antimesenteric borders were approximated and one fork of the GIA was inserted into each bowel lumen. Two double staggered staple lines joined the bowel walls, while simultaneously the knife assembly in the GIA divided between the two staple lines creating a stoma. The mesentery was adapted with 2 single sutures (Catgut 5-0^R). At the cranial end of the now one sided blind self-emptying pouch a cannula was placed according to Kik *et al.*[3], after which the abdomen was

closed.
Antibiotics (Trimetoprim-sulfadiazine, 80 mg Na. sulfadiazine 400 mg, 1ml/30 kg) (Tribrissen^{R} Coopers Agrovet B.V., Weesp, The Netherlands) and analgetics (flunixine-meglumine 2 mg/kg, intramuscularly, (Fynadine^{R}, Mycofarm Nederland b.v., De Bilt, the Netherlands)) were administered immediately after surgery and during 5 days and 3 days post-operatively, respectively.

Manufacture of the cannula

The cannula designed to penetrate the abdominal wall consists of a transmural tube with an attached intra-intestinal pair of wings. The construction is composed of surgical non toxic quality silicone rubber tubing (SR 30, Talas B.V., Ommen, The Netherlands) with an inner diameter (ID) of 150 mm and an outer diameter (OD) of 200 mm. It has a length of 10 cm while the wings extend 3 cm to either side. The tube is connected to the wings at an angle of 45° with medical grade silicone adhesive (Raumedic Adhesive SI, Talas B.V., Ommen, The Netherlands) that is applied in successive layers.
A plug for the transmural tube is prepared by filling the lumen of a length of narrower silicone tubing (ID 100 mm, OD 150 mm) with silicone adhesive (Mastic-Silicone, Perfecta Chemie B.V., Goes, The Netherlands). A ring of tubing is mounted at the external end of the plug in order to prevent it from sliding inwards. Finally, the plug is trimmed to fit the transmural tube accurately. Acetate is liberated during polymerization of the adhesive, and therefore surgical implantation should not be performed within 24 hours following construction. After surgical implantation in the intestine and exteriorization through the abdominal wall, the cannula is secured externally by a ring with a wide collar. This ring should also be prepared at least one day prior to surgery. The ring consists of silicone tubing with ID 190mm and OD 250 mm. The collar is composed of two concentric circular silicone bands with a diameter of 200 and 400 mm, respectively (SR 4 H, Talas B.V., Ommen, The Netherlands), that are attached to the ring at an angle of 45°. This permits accurate fitting of the collar to the cannula without compressing the tissues.

Obtaining jejunal mucosal biopsies

The first jejunal samples were taken on the day of surgery. From 10 days postoperatively, mucosal biopsies were taken with a slightly modified Lameris blind-biopsy tube for adults, nr. 546 (Lameris, Medical Equipment, Utrecht, The Netherlands)[3].

Subsequently, during a 6-week period mucosal biopsies were taken twice a week. On those occassions four biopsies were taken. Two biopsies obtained around 40 cm from the opening of the cannula were used to determine the enzyme activity of aminopeptidase and sucrase-isomaltase. Histological

investigations were performed on biopsies taken at a distance of 35 cm and biopsies at 32.5 cm were examined electron microscopically.

In the sixth week of the experimental period, the mucosa in the pouch was examined by introducing a flexible fiberscope though the cannula (Olympus CF, type 1T, 10L, Tokyo, Japan). Photographs were taken of the mucosal wall (Olympus OM2 body; 400 Asa Ektachrome film).

Tissue processing for morphometric analysis

For morphometric analysis one biopsy per experimental day was fixed in 0.1 M phosphate-buffered 4% formalin, pH 7.3, dehydrated, and embedded in paraffin. Serial sections (5μm) were cut and stained with hematoxylin and eosin. Using these sections, the length of 10 well orientated villi and depth of 10 crypts were measured by means of a TEA Image Manager system (DIFA, Breda, The Netherlands). The villus/crypt ratio was calculated to indicate morphological changes of the mucosa during the experimental period.

Tissue processing for transmission electron microscopy

One biopsy per experimental day was fixed in 0.1 M cacodylate buffered 2.5% glutaraldehyde (pH 7.35, 440 mOsm) during 24 hours at 4°C, and subsequently rinsed in 0.1 M cacodylate buffer (pH 7.35). After postfixation with 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.35) for 16 h at 4°C, the biopsy was dehydrated in graded water-acetone mixtures and embedded in Epon-Araldite mixture. Ultrathin sections were stained with uranylmagnesium acetate and lead citrate and examined with a Philips EM410LS electron microscope at 60 kV.

Sucrase-isomaltase and aminopeptidase activities

Per experimental day 2 biopsies were frozen (liquid nitrogen) and stored at -70°C until analysis. Thereafter all procedures were performed at 4° C, unless mentioned otherwise.

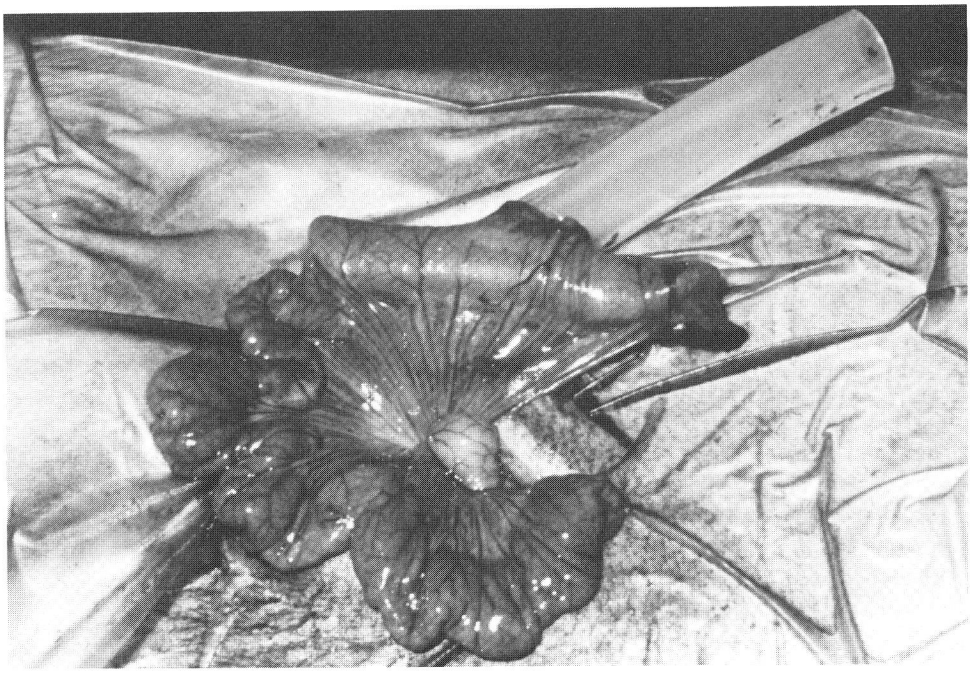
The biopsies were pooled in 1 ml of double-distilled water and sonified using a Branson sonifier (step 4; 2 x 10 sec; interval 30 sec.). After sonification an aliquot of the suspension was used for protein determination[9]. Sucrase-isomaltase (EC 3.2.1.48) activity was determined according to Messer and Dahlqvist[13], using sucrose (Serva, Heidelberg, Germany) as substrate. Aminopeptidase (EC 3.4.11.2) activity was measured according to Maroux *et al.*[12] using L-alanine-p-nitroanilide (Serva, Heidelberg, Germany) as substrate.

The specific enzyme activity was calculated as U/g of protein and expressed as the mean specific enzyme activity $\pm$ SEM.

Results

The animals recovered rapidly from the operation. During the entire experimental period the self-emptying blind pouch did not clinically influence the health of the animals.
When the plug of the transmural tube was opened, normal chyme poured out, indicating a normal supply of chyme in the pouch.

The exteriority of the self-emptying blind pouch and the position of the cannula are shown in figure 1. Fiberoptic examination of the mucosal wall at the end of the experimental period revealed a normal appearance of the mucosa



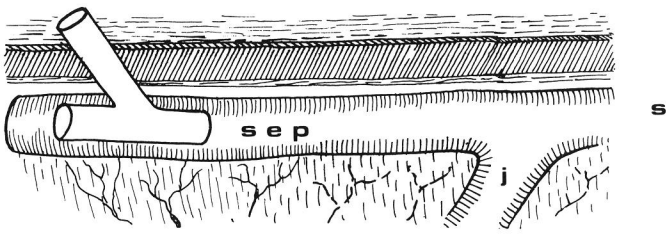


Figure 1a. The exterior of the Self-Emptying blind Pouch with the cannula at its blind end during surgery.
1b. Drawing of the Self-Emptying blind Pouch (SEP).
s = stomach, j = jejunum.

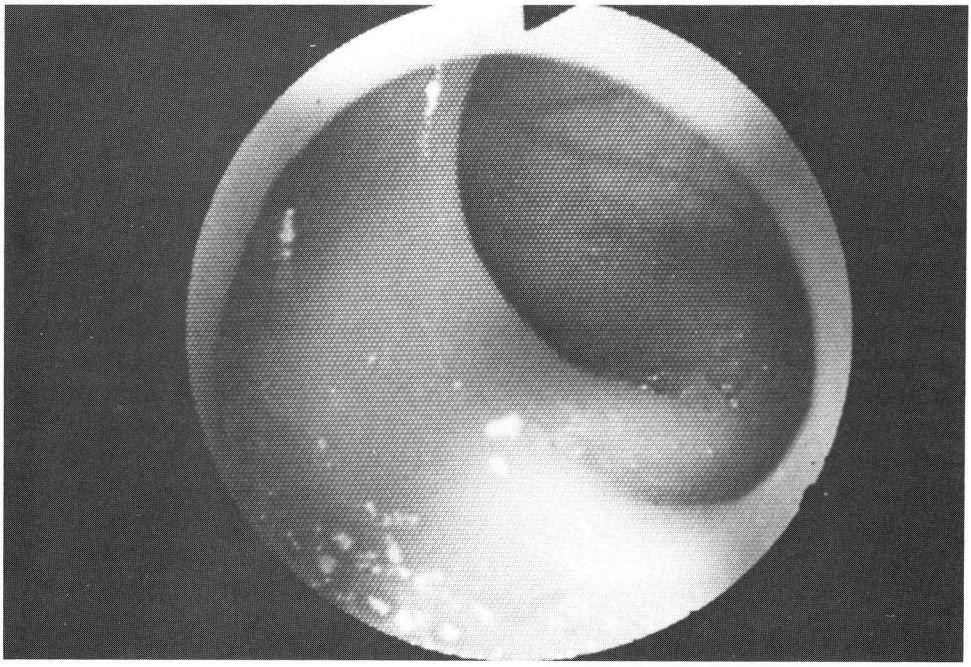
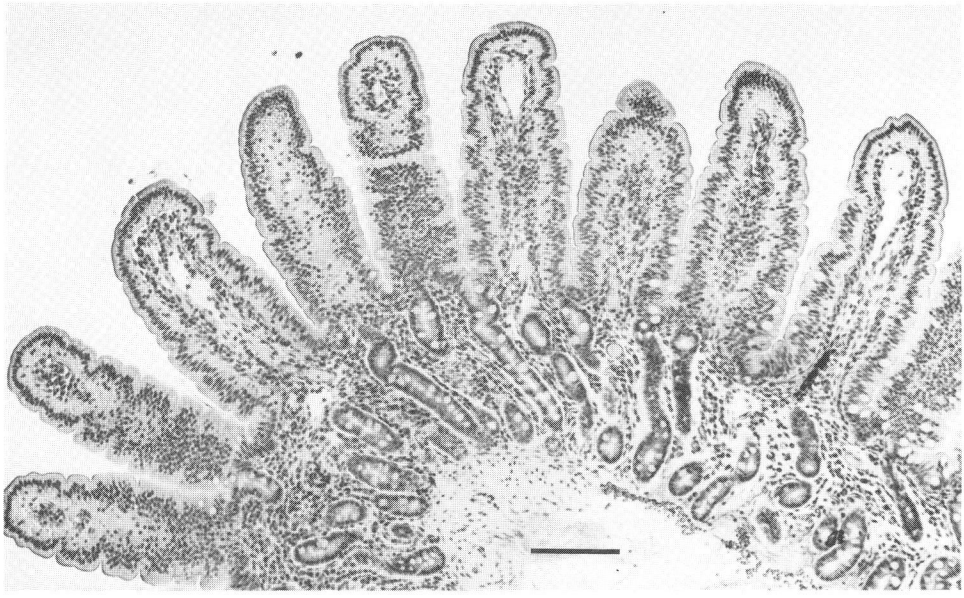


Figure 2. Fiberoptic observation of the mucosal wall in the Self-Emptying blind Pouch. The mucosa is of normal appearance. Blood vessels underlying the mucosa can be seen.



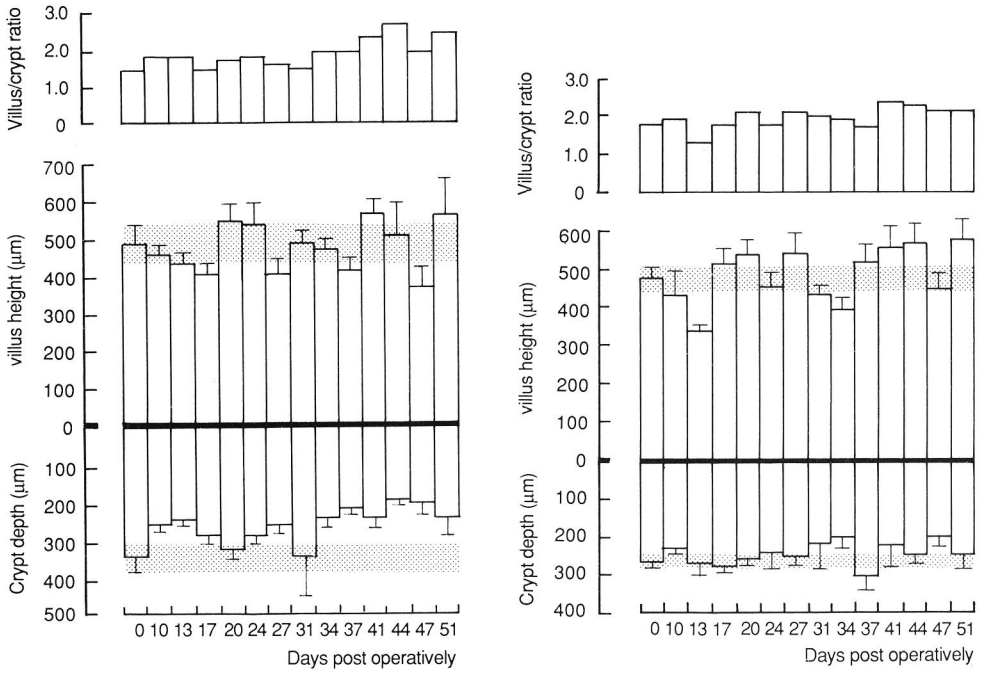


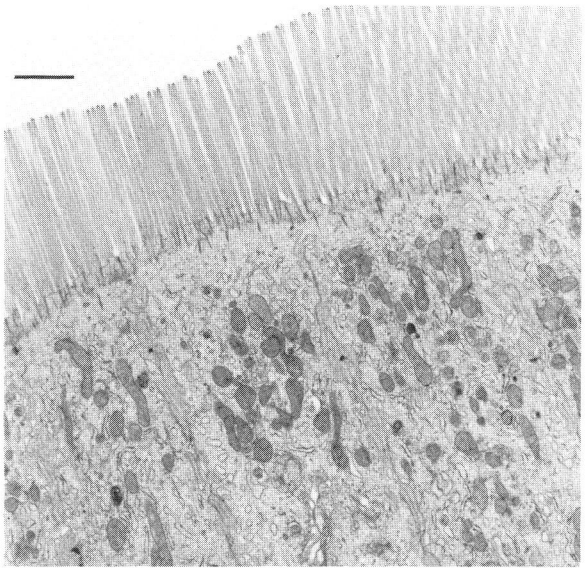
Figure 4. Mean villus height, crypt depth ± SD (µm), and villus/crypt ratio of the animals per experimental day during the experimental period.

of the jejunum in the pouch. Blood vessels underlying the mucosa can be seen (Fig. 2).

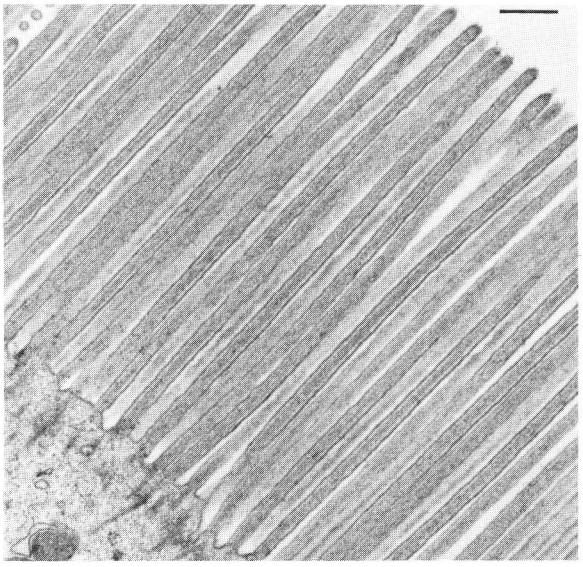
The histological appearance of a biopsy taken 20 days after surgery did not reveal any abnormalities (Figure 3). The villi were normally shaped. The columnar epithelium covering the villi had a normal aspect, and showed the so-called superfical scalloping. During the entire experimental period the brush border remained clearly visible at the apical end of the villus epithelial cells. The morphologic characteristics of the jejunal mucosa in the pouch are presented in figure 4.

The results of the morphometric analysis of the biopsies taken from the mucosa in the pouch and expressed separately for each pig showed a gradual decline in villus length at the beginning of the experimental period. Both animals recovered from this initial decrease. Neither the length of the villi, the depth of the crypts nor the villus/crypt ratio showed a pronounced tendency to decrease or increase during the entire experimental period. Most values did not differ

Figure 3. Histology of a section of a biopsy taken 20 days after surgery. The villi are of normal appearance and covered with a single layer of columnar epithelium. H.E. (bar is 6.9 mm).



5A



5B

Figure 5A. Electron micrograph of the apical surface of absorptive cells of a biopsy taken 20 days after surgery. The microvilli are even in length and regularly positioned. (Bar is 0.14 mm).
Figure 5B. Same, The cores of the microvilli consisting of bundled actin filaments are clearly seen to protrude into the apical cytoplasm. (Bar is 0.04 mm).

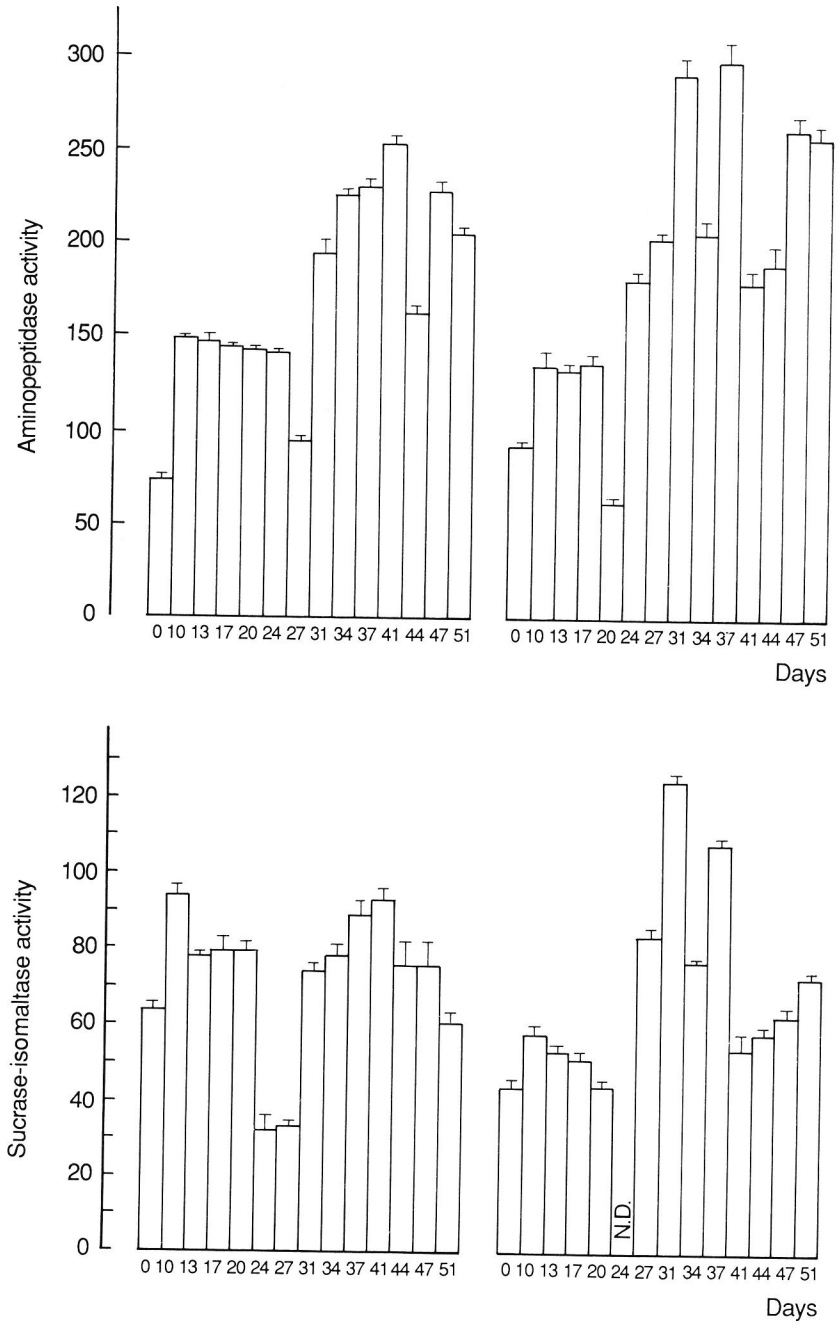


Figure 6. Sucrase-isomaltase and aminopeptidase activities in U/g of protein of the biopsies of the animals during the experimental period. Values give means ± SEM. n.d. = no data.

significantly from the mean value ± SD on the day of surgery (Figure 4).

Also at the ultrastructural level there were no indications of morphological changes in the jejunal mucosa of the pouch. During the entire experimental period the enterocytes were covered with microvilli, which were even in length and regularly positioned (Figure 5A). The core of the microvilli was clearly seen to protrude into the cytoplasm of the enterocytes. The cells contained organelles of normal appearance (Figure 5B).

The activity profile of the enzymes sucrase-isomaltase and aminopeptidase is presented in Figure 6.

In both animals the activity of aminopeptidase continued to rise gradually until the end of the period. Between the animals the aminopeptidase activity of the biopsies taken at the same point of time varied. Both activity profiles of aminopeptidase displayed a decrease postoperatively.
In comparison with the enzyme activity profile of aminopeptidase the sucrase-isomaltase activity showed the same tendency.

Discussion

The presence of a self-emptying blind pouch in the jejunum of the pig does not seem to interfere clinically with the health of the animals during the entire experimental period. The pouch itself is supplied with a normal chyme.

Apart from the gradual decline of the villus length of the mucosa in the pouch at the beginning of the experimental period, the morphological characteristics of the jejunal mucosa in the pouch fluctuated around the mean value on the day of surgery. No pronounced variances could be measured or calculated. The initial decrease in villus length may be attributed to surgery during which the pouch with its cannula was implanted. A comparable decrease was observed by Van Weeren-Keverling Buisman *et al.*[15] after inplanting a cannula in the small intestine of the calf.

The ultrastructure of the enterocytes of the mucosa in the pouch did not change during the experimental period. In particular, attention has been paid to the morphology of the microvilli, since the integrity of the microvilli is essential for enterocyte functioning. Distinct lesions in microvilli resulting in disruption could not be observed. The length of the microvilli was not liable to alterations.

The differences in enzyme activities of sucrase-isomaltase and aminopeptidase between one pig and another and during the experimental period in the same pig are most probably due to biological variation. Except for this variation the brush-border enzyme activities rose steadily in time. The levels of sucrase-isomaltase continue to rise until maturity in pigs[11] and an age-related increase in sucrase-isomaltase has been observed in rats[4]. For that reason the established increase in activities of sucrase-isomaltase as well as aminopeptidase in the biopsies taken from the mucosa of the pouch also demonstrate an age-related

increase.

We have demonstrated that the presence of a self-emptying blind pouch in the jejunum of piglets does not modify the mucosa in the pouch morphologically or functionally. For that reason the self-emptying blind pouch represents a most suitable model to study the effects of deleterious agents on the structure and function of the jejunal mucosa in the pig. The temporary decrease in villus length and brush border enzym activity renders this model useful beginning 2½ weeks postoperatively.

Acknowledgements

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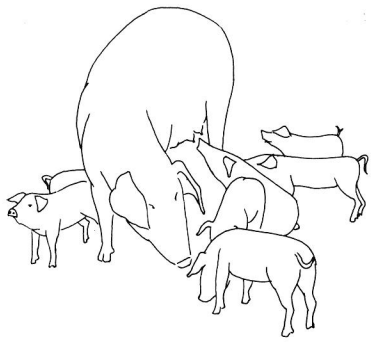
CHAPTER VIII

EFFECTS OF *PHASEOLUS VULGARIS* ISOLECTINS ON THE STRUCTURE AND FUNCTION OF THE SMALL INTESTINAL MUCOSA IN THE SELF-EMPTYING BLIND POUCH MODEL IN PIGLETS



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Summary

Specific pathogen free piglets were surgically provided with a jejunal one side closed self-emptying blind pouch. At the blind end a cannula was fitted. Through this cannula biopsies from the jejunal mucosa were taken with a blind-biopsy tube. Biopsies were taken at surgery and from 10 days post operatively at regular intervals during 2½ weeks before and after administration of a single dose of Phaseolus vulgaris (red kidney bean) isolectins E4 and L4 through the cannula. The morphology of the biopsies was studied by light and electron microscopy. The activity of sucrase-isomaltase was studied biochemically.

During the first 2½ weeks postoperatively the jejunal mucosa in the pouch did not change in morphologic and functional characteristics.

After administration of Phaseolus vulgaris isolectin-E4 the v/c ratio declined significantly. The glycocalyx was thickened, the microvilli were shortened and irregularly positioned, and numerous microvillous vesicles were tied off. Forty-eight hours after the administration electron microscopically the effects were still visible. The activity of sucrase-isomaltase showed initial decline but recovered rapidly. The changes were restored 4 days after the administration.

The effects of Phaseolus vulgaris isolectin-L4 were similar but less abundant.

Introduction

Lectins, which are characterized by their unique ability to bind to specific sugars or glycoconjugates, are ubiquitously present in legume seeds consumed by man and his farm animals[6,18,23,24]. Consumption of raw or poorly heat-treated red kidney beans (Phaseolus vulgaris) leads to an acute intestinal syndrome in man, consisting of nausea, vomiting, and diarrhea[5,23]. The presence of biologically active lectins in the small intestine may lead to a series of complex events which culminate in severe growth depression, emaciation, and ultimately death of the animals[19].

Untill now the effects of different lectins were studied in intact conventional animals, e.g. rats and pigs[1,9,12-16,20,30], in cultures of human colon carcinoma Caco-2 cells[2,7], and in pig small intestinal mucosa in organ culture[10]. In the feeding experiments using foods containing *P. vulgaris* beans or purified P. vulgaris agglutinin (PHA), atrophy and blunting of villi occurred in association with elongation of the crypts, shortening and disruption of the microvilli, and desquamation of enterocytes[1,9,12,20]. The activities of sucrase-isomaltase and aminopeptidase were reduced[10]. Addition of purified *P. vulgaris* isolectins to the human colon carcinoma Caco-2 cells induces shortening of microvilli and increased synthesis of (glyco)proteins of the cells[7]. Organ culture of pig jejunal mucosa in the presence of isolectins from *P. vulgaris* revealed shortening of the villi in combination with shortened and disrupted microvilli[10].

P. vulgaris agglutinin comprises a family of five glycoproteins, that are isolectins which contain various proportions of the L (mitogenic) and E (erythro-agglutinating)subunits[4,17]. To single out the direct effects of *P. vulgaris* isolectins E4 (PHA-E4, 4 erythro-agglutinating sububits) and L4 (PHA-L4, 4 mitogenic subunits) on the structure and function of the small intestinal mucosa *in vivo* in pigs, these isolectins were administered through a cannula in the self-emptying blind pouch (SEP) model[11]. In this model only small amounts of the isolectins are necessary, and the isolectins are not directly influenced by the stomach.
The aim of this study was to investigate the effects of PHA-E4 and PHA-L4 on the morphology and function of the small intestinal mucosa in the SEP model.

The morphologic and functional state of the mucosa in the pouch was studied before and after administration of the isolectins by histometric analysis of the villus length, crypt depth, and number of mitoses; by ultrastructural investigation of the microvilli; and by biochemical analysis of the specific brush border membrane enzyme, sucrase-isomaltase.

Experimental design

Four female SPF piglets (CDI, Lelystad, The Netherlands) 7 weeks of age,

were individually housed in overpressure stables and fed a lectin-free diet[11]. Water was allowed ad libitum.

After 24 hours of fasting the animals were surgically provided with a self emptying blind pouch about 50 cm in length, in the jejunum, according to Kik *et al*[11]. In short, a one side closed self emptying blind pouch with intact blood vessel and nerve supply, with a cannula at its blind end was established in the jejunum of the piglets 4.0 m cranial to the ileocaecal ligament. The animals were allowed to recover 10 days following surgery. From then on, mucosal biopsies were taken twice a week during two weeks to study the morphological and functional state of the jejunal mucosa in the pouch[8]. Seventeen and 23 days after surgery, in each 2 animals, 50 ml of Trowell's T-8 medium[27] with 50 μg of PHA-E4 or PHA-L4 per ml (E-Y laboratories Inc., San Mateo, California, U.S.A.) were administered through the cannula. Five, 24 and 48 hours after administration mucosal biopsies were collected. Further biopsies were taken twice a week during 2 weeks following the administration of the isolectins.

Each time 4 biopsies were taken, the first 2 around 25 cm from the opening of the cannula for biochemical determination of the activity of sucrase-isomaltase. One biopsy was taken at 20 cm from the opening of the cannula for morphometric analysis and one at 17.5 cm for electron microscopy.
Biopsies for immunocytochemical determination of the binding of the isolectins were additionally taken 22.5 cm from the cannula, 5, 24, and 48 hours after the administration of the isolectins.

Tissue processing for histometric analysis

For histometric analysis the biopsies were fixed in 0.1 M phosphate-buffered 4% formalin at pH 7.3, dehydrated, and vertically cut in two. Subsequently, the pieces were carefully placed on their cut edge and embedded in paraffin. Serial sections (5μm) were made and stained with haematoxylin and eosin. Using these sections, the length of ten well oriented villi and the depth of ten crypts were measured by means of a TEA Image Manager system (DIFA, Breda, The Netherlands). The villus/crypt ratio was then calculated to indicate the extent of morphological changes of the mucosa. Fifteen crypt columns were used to determine the number of mitoses (meta- and anaphases) per crypt.

Tissue processing for transmission electronmicroscopy

The biopsies for electron microscopy were fixed in 0.1 M cacodylate buffered 2.5% glutaraldehyde (pH 7.35, 440 mOsm) during 24 hours at 4° C, and subsequently rinsed in 0.1 M cacodylate buffer (pH 7.35). After postfixation with 1% OsO4 in 0.1 M cacodylate buffer (pH 7.35) for 16 h at 4° C, the biopsy was dehydrated in graded water-acetone solutions and embedded in Epon-Araldite mixture. Ultrathin sections were stained with uranylmagnesium acetate

and lead citrate and examined with a Philips EM410LS electron microscope at 60 KV.

Tissue processing for immunohistochemistry

The biopsies for immunohistochemistry were immediately fixed in sublimate-formalin (5% $HgCl_2$: concentrated acetic acid : 40% formaldehyd / 17:1:2). After 2 hours of fixation, the biopsies were dehydrated, embedded in parrafin and cut at 5μm. The paraffin sections were desublimated (5 min in 0.5% I_2 in 70% (v/v) ethanol and 5 min in 2.5% sodiumthiosulphate) preceding the immunoperoxidase staining method (PAP) according to Vos *et al.*[29] Commercially available rabbit anti-PHA-E4 and anti-PHA-L4 immunoglobulins, swine anti-rabbit immunoglobulins (Sanbio, Uden, The Netherlands), and the PAP-complex (Dakopatts, Glostrupp, Denmark) were used. In the control sections, the antilectin was replaced with buffer or normal serum.

Sucrase-isomaltase acitivity

The biopsies for biochemistry were frozen in liquid nitrogen and stored at -70° C until analysis. All subsequent procedures were performed at 4° C, unless mentioned otherwise. The biopsies were pooled in 1ml of double-distilled water and sonified twice for 10 s (interval 30 s.). After sonification an aliquot of the suspension was used for protein determination[21]. Sucrase-isomaltase (EC 3.2.1.48) activity was determined according to Messer and Dahlqvist[22], using sucrose (Serva, Heidelberg, Germany) as substrate. The specific enzyme activity was calculated as U/g of protein and expressed as the mean specific enzyme activity $\pm$ SEM.

Statistical analysis

The statistical significance between means was analysed using the paired student's t-test.

Results

During the first 2½ weeks after surgery normal chyme poured out of the cannula when opened. Twenty-four hours after administration of the PHA-E4 a more slimy and larger amount of chyme came out of the cannula. The animals were slower, and their food intake was less than usual during 24 hours after the administration of PHA-E4, whereas the PHA-L4 treated animals did not show any of these clinical signs.

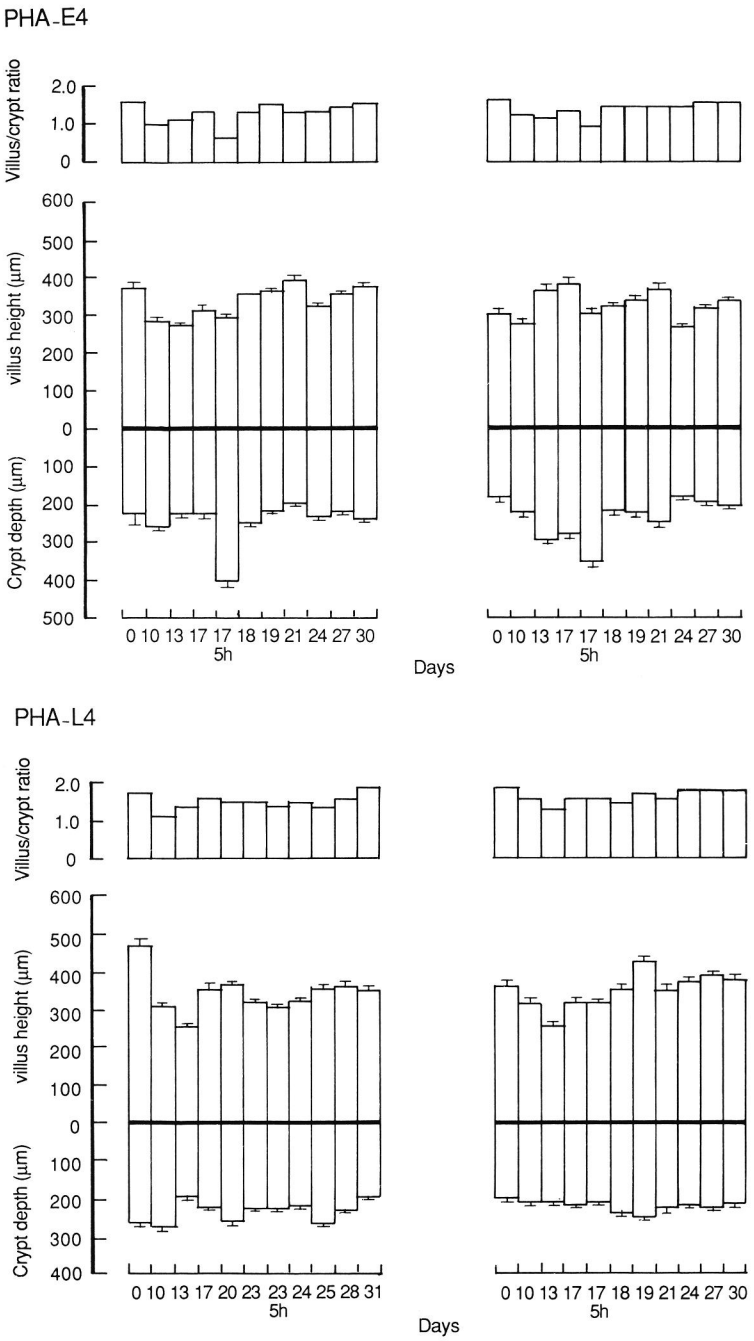


Figure 1. Morphologic characteristics of the jejunal mucosa of the SEP, before and after the administration of the PHA-E4 and PHA-L4, respectively. Villus length, crypt depth, and villus/crypt ratio are shown. Values give mean ± SEM.

The results of the histometric analysis are summarized in figure 1.
Five hours after the administration of PHA-E4 all affected animals showed a decrease in villus length, whereas an increase in crypt depth occurred. The calculated villus/crypt ratio was lower. Only the increase in crypt depth and decrease of villus/crypt ratio were statistically significant. About 4 days after the administration of the isolectins the villi and crypts gradually regained their

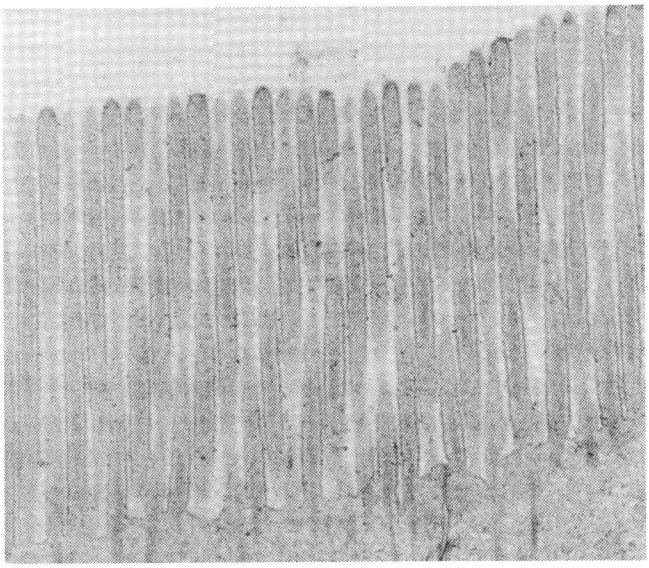


Figure 2. Electron micrograph of the brush border of a control biopsy 17 days after surgery. Note the densily packed and regularly positioned microvilli. x 22750.

normal length.

In the PHA-L4 treated animals no changes occurred in villus length, crypt depth and villus/crypt ratio within 5 hours after the administration.

The number of mitoses remained practically the same throughout the entire experimental period for both isolectins.

The microvilli of a control biopsy are depicted in figure 2. Five hours after the administration of the PHA-E4 the microvilli were shortened, the glycocalyx was thicker and had a more fuzzy appearance. Some microvillar vesicles were tied off (fig. 3). At 24 hours larger numbers of microvillar vesicles were tied off (fig. 4). Forty-eight hours after the administration complete disappearance of microvilli frequently occurred, the remaining microvilli being shortened and irregularly positioned (Fig. 5). With PHA-L4 the effects were less than those produced by PHA-E4. From about 4 days after the administration of the isolectins the enterocytes had recovered, the microvilli had regained their normal length and were long and slender again.

Immunocytochemical binding of PHA-E4 and PHA-L4 could be detected in

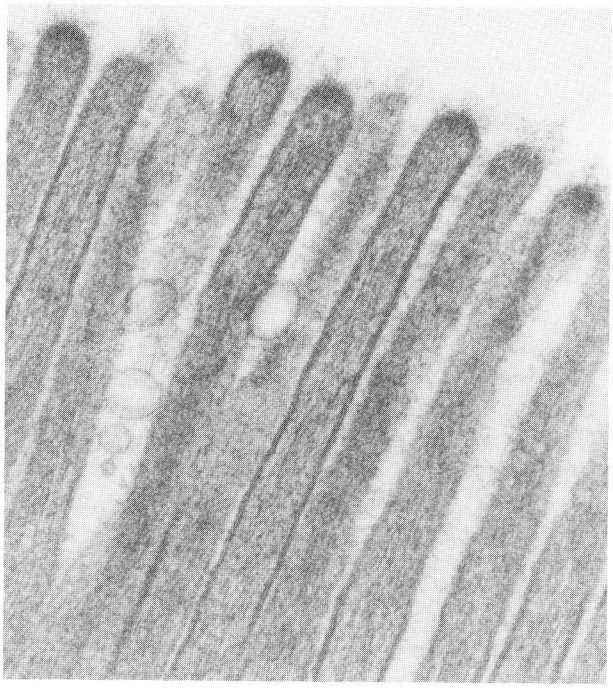


Figure 3. Electron micrograph of the brush border of a mucosal biopsy 5 hours after the administration of PHA- E4. Note the thickened glycocalyx and the presence of a few microvillus vesicles. x 71250.

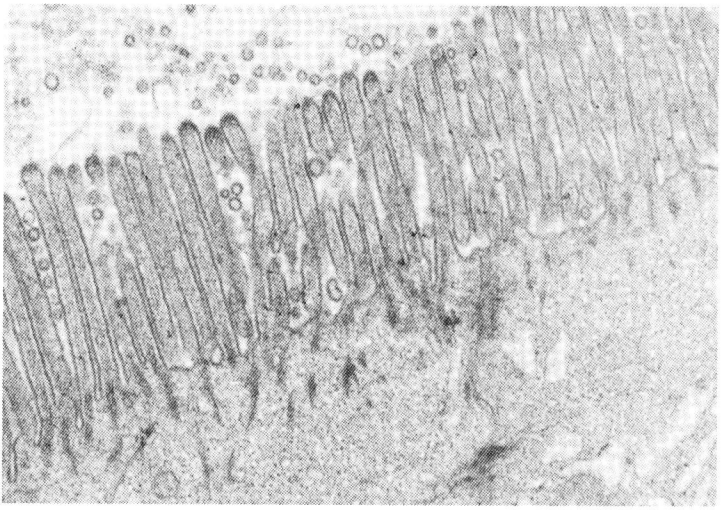


Figure 4. Electron micrograph of the brush border of a mucosal biopsy 24 hours after the administration of the isolectin-E4. Note the large number of tied off microvillus vesicles, irregularity, and shortening of the microvilli. x 22750.

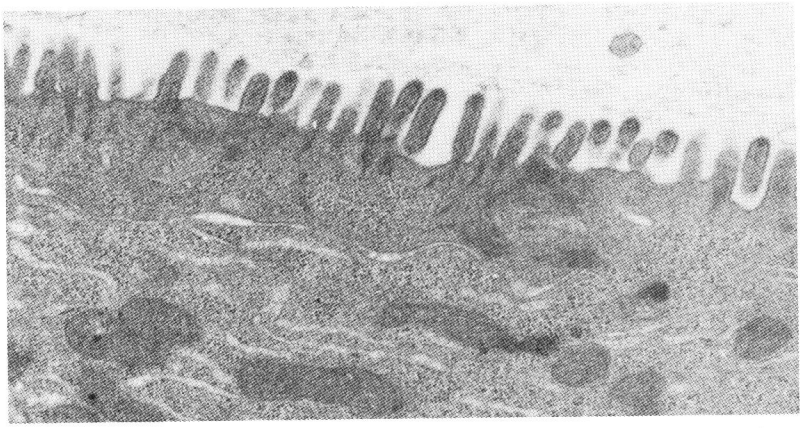


Figure 5. Electron micrograph of the brush border of a mucosal biopsy 48 hours after the administration of PHA-E4. The microvilli are irregularly positioned, shortened and decreased in numbers. x 22750.

the brush border regions of the villus enterocytes 5 and 24 hours after the administration of the isolectins. After 48 hours binding of the isolectins was hardly detectable. In all control sections, where the antilectin was replaced with buffer or normal serum, the sections showed no staining.

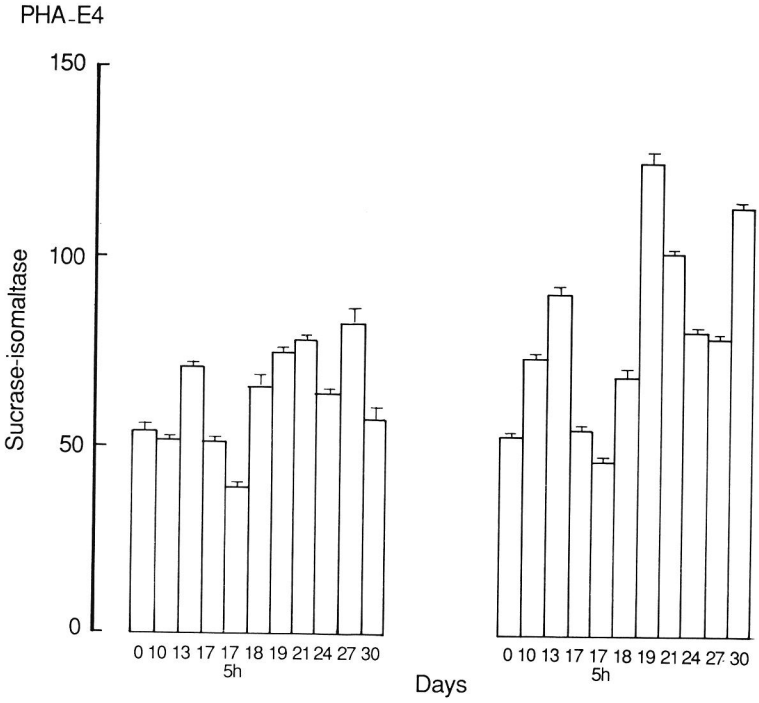
The activity of sucrase-isomaltase in the PHA-E4 treated animals was decreased, and then rose steadily throughout the experimental period. In the PHA-L4 treated animals the activity showed a statistically significant decline 5 hours after the administration, followed by a rise (Fig. 6).

Discussion

The objective of this study was to investigate whether administration of PHA-E4 and PHA-L4 in the SEP model induces morphological and functional changes.

Within the first 13 days following the establishment of the Self-Emptying blind Pouch in the jejunum of the piglets the mucosa showed a temporary decline in villus length and activity of sucrase-isomaltase as reported earlier[11] which is probably due to surgery itself[28]. Apart from this decline the mucosa in the self-emptying blind pouch did not change as far as morphological and functional characteristics are concerned.

Five hours after the administration of the PHA-E4 there was a decline in villus length combined with an increase in the crypt depth which is in agreement with the findings of Kik *et al.*[9] when raw *P. vulgaris* beans were fed to pigs. A statistically significant decrease in villus length was seen in organ culture of pig


PHA-E4
150
100
50
0
Sucrase-isomaltase
0 10 13 17 17 18 19 21 24 27 30
5h
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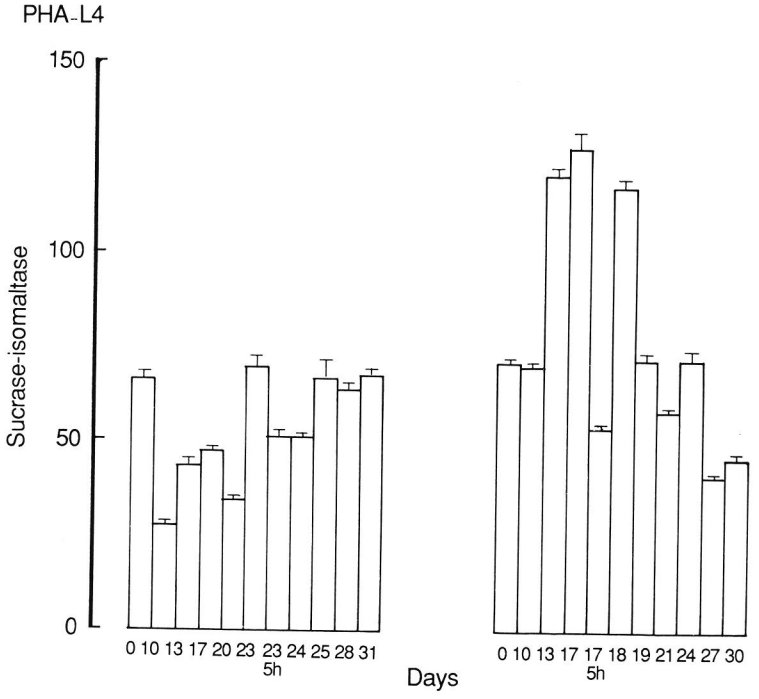
Days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small intestinal mucosa incubated with isolectins[10]. After the administration of PHA-L4 light microscopical changes were minimal, which does not agree with the findings in organ culture.

Binding of both the isolectins was demonstrated by immunocytochemistry. Five and 24 hours after the administration binding occurred to the brush border of the villus epithelial cells. King *et al.*[15] also demonstrated the binding of *P. vulgaris* agglutinin to villus epithelial cells of the jejunum of pigs.

In this study thickening of the glycocalyx was noticed, starting 5 hours after the administration of PHA-E4, followed by the tying off of numerous microvillus vesicles, irregularity, shortening, and disappearance of microvilli. These microvillus abnormalities are also described by Rossi *et al.*[25] when rats were fed purified red kidney bean lectins. They suggest that the abnormalities are the morphological manifestation of a change in the apical plasma membrane protein content and turnover, caused by the lectins. Thickening of glycocalyx was also noted by Egberts *et al.*[3] when rats were treated with methotrexate, and was considered an a-specific reaction to damage of the intestinal epithelium. The same alteration was seen after administration of PHA-L4, but less abundant[10].

The decrease in activity of brush border enzymes sucrase-isomaltase is also described when rats or pigs were fed whole raw red kidney beans[9,26]. The lectin-induced shedding of the brush border membrane as vesicles and the shortening of microvilli may account at least partly for the loss of sucrase-isomaltase activity.

In summary, we have shown that the administration of PHA-E4 and PHA-L4 in the SEP model leads to changes in the structure and function of the small intestinal mucosa comparable to those when raw red kidney beans or *P. vulgaris* were given to animals orally. Thus, rendering the SEP model useful as an *in vivo* model in the pig, when the effects of small amounts of purified lectins have to be investigated.

Acknowledgements

The authors would like to thank Mr. H. Rutgers for perfect taking care of the animals, in assisting during surgery and taking of the biopsies.

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Figure 6. Activity of sucrase-isomaltase in U/g of protein in the jejunal samples before and after administration of PHA-E4 and PHA-L4.

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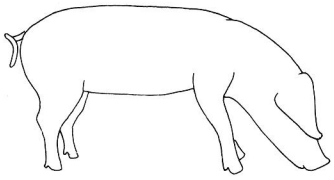
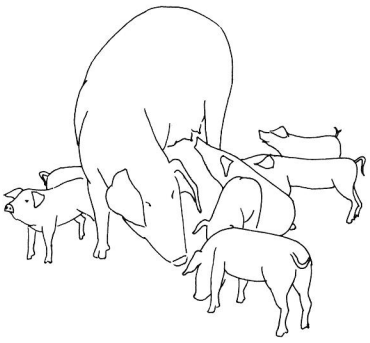
CHAPTER IX





SUMMARIZING DISCUSSION

Legume seeds, e.g., *Phaseolus vulgaris* (red kidney bean), *Glycine max* (soya bean), *Pisum sativum* (pea), and *Vicia Faba* (field bean), are used world-wide as food for human beings and their farm animals[10]. However, the presence of a number of antinutritional factors, such as lectins, tannins, and protease-inhibitors in untreated beans and peas, are a disadvantage to legume consumption[9].

In this thesis effects of lectins in legume seeds, particularly *Phaseolus vulgaris* (red kidney bean) agglutinins, on intestinal epithelial metabolism, structure and function *in vivo* and *in vitro* and their pathogenesis are described. It forms a part of a larger multidisciplinary study in which aspects of digestive physiology[8] and food-technology[15] have also been investigated. Moreover, it formed the foundation for the development of the Functional Lectin Immuno Assay (FLIA)[7].

The literature concerning the interaction of plant-lectins with, and their effects on the small intestinal epithelium has been reviewed in **chapter II**.

Lectins are generally defined as (glyco)proteins of non-immuno-globulin nature[7] with the capacity of reversible binding to carbohydrate moieties of glycoconjugates, such as that present in the glycocalyx of the intestinal epithelium[4]. In principle the lectins are heat-sensitive, but nevertheless heat processing of beans and peas is not always effective in abolishing the (patho)biological activity of the lectins. Moreover, heat processing is an expensive factor in the production of animal feed[11,15].

The toxicity of *Ricinus communis* agglutinin (RCA) has already been known since 1888, when Stillmark discovered a hemagglutinating substance in the aqueous extracts of *Ricinus* seeds[13]. It is now generally acknowledged that predominantly *P. vulgaris* agglutinin (PHA) is responsible for the toxicity in monogastric species, man or animal, of diets based on raw red kidney bean[2].

Many lectins, especially PHA, are resistant to gut proteolysis. Biologically active lectins, when they reach the intestinal tract, bind to their specific mono- or oligosaccharides, present in the glycoconjugates of the intestinal glycocalyx. This binding evokes a number of largely unknown events, eventually leading to changes in the digestive and absorptive functions of the small intestine[1,12,17].

Much research has been conducted on the effects of lectins in different laboratory animals. The feeding of lectin-containing seeds or the administration of purified lectins may lead to damage of the small intestinal mucosa, diarrhoea, poor growth, emaciation, and even death of the animals[1,6]. However, there is no assurance that the *in vivo* toxicity of lectins in mice and rats can be extrapolated to other species of animals because of species differences[8]. Life weight gain, feed conversion efficiency, and the weight of the spleen and thymus were markedly reduced in piglets fed raw *P. vulgaris* , whereas there was little or no

effect on these parameters in rats or chickens[8].

Little is known about the effects of addition of lectins to diets of farm animals such as pigs on the structure and function of the small intestinal mucosa. Therefore, the experimental studies were started with *in vivo* addition of raw *P. vulgaris* beans to diets of piglets.

In **chapter III** the effects of feeding crude *Phaseolus vulgaris* beans to pigs on the structure and function of the small intestinal mucosa are described. Feeding of raw *P. vulgaris* beans to piglets leads to scouring, wasting, and diminished growth of the animals. Grossly the mucosa was covered with a larger amount of mucus. Microscopically the small intestinal mucosa was characterized by atrophy and blunting of the villi, in association with elongation of the crypts and increased mitotic activity. Compared to those in controls, goblet cells were significantly decreased in the villi but markedly increased in the crypts. The activity of the brush border enzymes aminopeptidase and sucrase-isomaltase was significantly decreased. The severe mucosal damage may interfere with the digestive and absorptive functions of the small intestine. Besides a diminished digestion of proteins, Huisman[8] found a marked loss of endogenous nitrogen in piglets after feeding of red kidney beans.

In order to gain more insight into the pathogenesis of the *in vivo* changes of the small intestinal mucosa two *in vitro* models were used, the Caco-2 cell line (**chapter IV**) and the pig jejunal mucosa in organ culture (**chapter V**).

To study whether the *in vivo* effects are directly related to the action of lectins, without any influence of the intestinal microflora or immunological responses[14], and whether there is a difference between isolectins, experiments were carried out adding purified *P. vulgaris* isolectins to the differentiated human colon carcinoma Caco-2 cell culture. The binding of *P. vulgaris* isolectins to differentiated human colon carcinoma Caco-2 cells and their effect on cellular metabolism and microvillus structure are described in **chapter IV**.

Incubation of the cells with ferritin-conjugated isolectins revealed that, as indicated by the number of ferritin particles, PHA-E4 bound to the microvilli to a greater extent than PHA-L4. Intracellularly ferritin-conjugated isolectins were detected over apical vesicles. Determination of the lectin binding sites by the enzyme-linked lectin sorbent assay (ELLSA) per mg of brush border membrane of Caco-2 cells revealed slightly more binding sites for PHA-L4. Compared to PHA-E4 the dissociation constant of PHA-L4 was 4 times greater. The number of binding sites for *Pisum sativum* agglutinin (PSA), *Vicia faba* agglutinin (VFA) and Soybean agglutinin (SBA) is higher than for the PHA isolectins, but the strength of their binding is far less (unpublished data).

After incubation for 48 hours with PHA-E4 or PHA-L4 the relative incorporation of precursors for DNA, RNA, and (glyco)protein synthesis into the trichloro-acetic acid insoluble fraction of the Caco-2 cells was determined.

Both isolectins stimulated the incorporation of ^{14}C-thymidine and ^{3}H-glucosamine, whereas neither PHA-E4 nor PHA-L4 were able to influence the incorporation of ^{14}C-uridine. With respect to ^{3}H-fucose, ^{35}S-methionine and N-acetyl ^{3}H-mannosamine the stimulatory effect remained confined to PHA-E4. These incorporation experiments suggest that exposure to PHA-E4 leads to a stimulation of DNA and (glyco)protein synthesis in differentiated Caco-2 cells. The DNA content and cell viability did not change significantly with increasing lectin concentration. For that reason DNA synthesis most probably does not account for the increased incorporation of ^{14}C-thymidine. Whether the incorporation is due to an increased DNA repair is presently unknown. The relative incorporation of ^{14}C-uridine did not change. Therefore, the increased rate of (glyco)protein synthesis may be due to a translational rather than transcriptional control. The increased (glyco)protein synthesis corresponds with the augmented mucus production when rats[6] or piglets (**chapter III**) were fed *P. vulgaris* beans. Moreover, the increase may be related to the thickening of the glycoclyx as is found in the Self-Emptying blind Pouch model (**chapter VIII**). The PHA-L4 induced changes in cellular metabolism do not correlate with the hemagglutinating activity of this isolectin. Incubation of differentiated Caco-2 cells with PSA did not influence the incorporation of radioactive precursors for DNA and (glyco)protein synthesis. In Caco-2 cells incubated with VFA the incorporation of ^{3}H-glucosamine, ^{3}H-fucosa and ^{14}C-thymidine was inhibited, whereas the ^{14}C-uridine was not influenced (unpublished data).
Compared to control, and PHA-L4 incubated Caco-2 cells, the microvilli of PHA-E4 incubated cells were shortened significantly. Near the brush borders of PHA-E4 and PHA-L4 incubated Caco-2 cells there were increased numbers of microvillus vesicles, covered with ferritin conjugated isolectins. The presence of these vesicles may reflect an increased turnover of the microvillus membrane[12]. In general receptor-ligand binding induces rapid cellular changes in pH, calcium concentration, and phosphorylation of cytoskeleton-associated proteins, disturbing the assembly or organization of actin filaments. The increase of globular actin may be responsible for the shortening, blebbing, and vesiculation of the microvilli[16]. In SBA incubated Caco-2 cells the shortening of microvilli appeared to be accomplished by changes in the ratio globular:filamentous actin[3]. Therefore, it is tempting to suggest that the PHA-E4 and PHA-L4 induced alterations of the microvilli have to be attributed to actin cytoskeletal lesions as well. PSA and VFA did not change the microvillus length significantly (unpublished data). Low concentrations of PHA-E4, PHA-L4, PSA and VFA stimulated the specific activity of sucrase-isomaltase, whereas the activity was decreased by higher concentrations of mentioned lectins (unpublished data).
After incubation with heat-inactivated PHA-E4, the described effects did not occur.

Since PHA-E4 and PHA-L4 were tested at the same concentration, we conclude that PHA-E4 is more effective in inducing changes than PHA-L4.

In summary, we conclude that after binding of PHA-isolectins to the brush border membrane receptors of differentiated Caco-2 cells and internalization of bound isolectins, PHA directly induces a number of metabolic and structural changes in intestinal epithelial cells.

The effects of PHA-E4 and PHA-L4 on jejunal mucosa of SPF piglets in organ culture have been described in **chapter V**. The binding of PHA-E4 and PHA-L4 revealed by immuno-electron microscopy was only demonstrated to the microvilli of the villus cells and not those of the crypt cells. No differences were observed concerning the distribution of the isolectins. In the explants, cultured for five hours in the presence of the isolectins, the enterocyte height and the villus length were decreased, and a lower villus/crypt ratio was calculated. PSA and VFA treated explants showed a decrease in villus length without influence on the crypt depth, and the calculated villus/crypt ratio was lower (unpublished data). At the ultrastructural level, the microvilli of PHA treated explants were shortened and irregularly positioned. Clusters of small vesicles, tied off from the microvilli, were seen in higher numbers when compared to control explants. The vesicles were covered with immunogold-labeled isolectins. These alterations were more severe in the PHA-E4 treated explants than in the PHA-L4 treated ones. In PSA treated explants the microvilli were somewhat irregularly positioned and some microvillus vesicles were tied off (unpublished data). The activity of sucrase-isomaltase was decreased in PHA-E4 cultured explants, whereas the enzyme activity did not change in PHA-L4 incubated explants. PSA induced a decline in activity of sucrase-isomaltase, whereas addition of VFA didnot change its activity (unpublished data).

The shortening of the villi, alteration of the microvilli, and decreased activity of sucrase-isomaltase are similar to the lesions *in vivo* in pigs (**chapter III** and **VIII**). The *in vitro* responses of pig mucosal explants to PHA isolectins indicate that this model is suitable for the determination of toxic effects of lectins and for further *in vitro* studies on the pathogenesis of the lectin induced direct lesions.

In **chapter VI** a small intestinal mucosal biopsy technique in cannulated piglets is described. This technique enables repeated sampling of small intestinal mucosa. About 80 cm caudal to the ligament of Treitz a cannula was placed in the intestinal lumen. Through this cannula mucosal biopsies were gathered with a Laméris blind-biopsy tube for adults, nr 546. The mucosal biopsies were of good morphological quality. With this method less animals are needed in an experimental study, since an experimental animal can also serve as its own control, before and after the experimental treatment. Moreover, the biopsies may be used for organ culture of small intestinal mucosa (unpublished data). Finally, this technique enables the sampling of biopsies in the Self-Emptying blind Pouch model as described in **chapter VII**.

In **chapter VII** the development of the Self-Emptying blind Pouch (SEP) model in the jejunum of piglets is described. This model was created for reasons of being able to study the effects of small amounts of isolectins on the structure and function of the small intestinal mucosa. Piglets were surgically provided with an one side closed Self-Emptying blind Pouch with intact blood vessel and nerve supply. At the blind end a cannula was fitted. Through this cannula mucosal biopsies were taken with a blind-biopsy tube. Biopsies were taken after 10 days post operatively, twice a week during 6 weeks, and studied morphologically as well as functionally. During this period, apart from a temporary postoperative decline in villus length and activity of the brush border enzymes sucrase-isomaltase and aminopeptidase, the jejunal mucosa in the pouch did not change with regard to morphological or functional characteristics.

Chapter VIII deals with the effects of PHA-E4 and PHA-L4 on the structure and function of the small intestinal mucosa in the Self-Emptying blind Pouch (SEP) model in SPF piglets. Through the cannula biopsies were taken after 10 days post operatively at regular intervals, during 2½ weeks before and after the administration of PHA-E4 and PHA-L4 through the cannula.

During the first 2½ experimental weeks the jejunal mucosa in the pouch did not change in morphological and functional characteristics, with the exception of a temporary postoperative decline in villus length and activity of sucrase-isomaltase.

Five hours after administration of PHA-E4 the villus length was decreased, the crypt depth increased, and the calculated villus/crypt ratio was lowered. About 4 days after the administration the villi and crypts regained their normal length. In the PHA-L4 treated animals the light microscopic changes were minimal. No change in mitotic activity of the crypt epithelium could be demonstrated.

Binding of the isolectins was demonstrated by immunocytochemistry. Five and 24 hours after the administration binding of both isolectins was confined to the epithelial cells of the villi and not to the crypts. After 48 hours binding was hardly detectable.

Electron microscopically, 5 hours after PHA-E4 administration higher numbers of microvillus vesicles were tied off, the glycocalyx was thickened and had a more fuzzy appearance. This phenomenon was also observed in rats fed PHA[12], and after injecting rats with methotrexate[5]. An explanation may be that as a response to the damage of the intestinal epithelium, the enterocytes may be triggered to overproduction of glycocalyx material[5]. Twenty-four hours after the administration, the microvilli were shortened and irregularly positioned, numerous microvillus vesicles were tied off. After 48 hours complete disappearance of microvilli often occurred, whereas the remaining microvilli were shortened and irregularly positioned. The activity of sucrase-isomaltase showed an initial decline but recovered rapidly. The effects were restored 4 days

afterwards. The effects of PHA-L4 were similar but less abundant.

In conclusion it can be stated that:

* *Phaseolus vulgaris* beans induce severe alterations of the small intestinal mucosa in piglets.
* In piglets PHA-E4 as well as PHA-L4 only bind to the villus epithelium and not to the crypt cells.
* PHA-E4 induces more severe alterations of the intestinal epithelium than PHA-L4.
* The lectin-induced alterations of the intestinal epithelium are at least partly caused by a direct interaction between the isolectins and the villus epithelium.
* Small intestinal mucosal biopsies of good quality can be obtained by a biopsy technique in cannulated piglets.
* The differentiated human colon carcinoma Caco-2 cells, the pig jejunal mucosa in organ culture, and the Self-Emptying blind Pouch are suitable models to determine toxic effects of small amounts of lectins on intestinal epithelium.
* The differentiated human colon carcinoma Caco-2 cells and the pig jejunal mucosa in organ culture are suitable *in vitro* models to study the pathogenesis of direct lectin-induced alterations of the intestinal epithelium.
* The cannulated pig and the Self-Emptying blind Pouch in piglets are suitable *in vivo* models to study the pathogenesis of indirect lectin-induced alterations of the small intestinal mucosa in pigs.
* The *in vivo* and *in vitro* models make it possible to reduce drastically the number of experimental animals needed for the investigation of the effects of lectins on the small intestinal mucosa and their pathogenesis.

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SUMMARY

The effects of lectins in legume seeds, particularly *Phaseolus vulgaris* (red kidney bean) agglutinins on intestinal cell metabolism, structure, and function in different *in vivo* and *in vitro* models and their pathogenesis are described.

The literature concerning the pathological effects of lectins in legume seeds on the structure and function of the small intestinal epithelium is reviewed in **chapter II**. After oral intake, intact plant lectins can reach the small intestinal lumen in an active form. Their binding to the mucosal surface evokes an increased synthesis of mucins and alteration of the small intestinal epithelium, resulting in a hyperregenerative villus atrophy and endogenous nitrogen loss. These changes ultimately can lead to less efficient feed conversion, diminished growth, scouring, wasting, and death.

Chapter III deals with the pathological effects of feeding of raw *P. vulgaris* beans on the structure and function of the small intestinal mucosa in piglets. The jejunal mucosa was characterized grossly as mucosal atrophy and microscopically as atrophy and blunting of the villi in association with elongation of the crypts and increased mitotic activity. Compared to those of controls, goblet cells were significantly decreased in the villi but markedly increased in the crypt region. The activity of aminopeptidase and sucrase-isomaltase was significantly decreased. These findings suggest that feeding of *P. vulgaris* beans reduce the digestive and absorptive capacity of the mucosa, resulting in diarrhoea, diminished growth, and weight loss.

In **chapter IV** the binding of kidney bean (*P. vulgaris*) isolectins to differentiated human colon carcinoma Caco-2 cells and their effect on cellular metabolism and microvillus structure are described. Incubation of the cells with ferritin-conjugated isolectins revealed that PHA-E4 bound to the microvilli to a greater extent than PHA-L4. Compared to PHA-E4 the dissociation constant of PHA-L4 was 4 times greater. After incubation for 48 hours with PHA-E4 or PHA-L4 the relative incorporation of precursors for DNA, RNA, and (glyco)protein synthesis into the trichloro-acetic acid insoluble fraction of the Caco-2 cells was determined. Both isolectins stimulated the incorporation of thymidine and glucosamine, whereas neither PHA-E4 nor PHA-L4 were able to influence the incorporation of uridine. With respect to fucose, methionine and N-acetyl-mannosamine the stimulatory effect remained confined to PHA-E4. Since PHA-E4 and PHA-L4 were tested at the same concentration, PHA-E4 is more effective than PHA-L4. Compared with those of controls and PHA-L4 incubated Caco-2 cells, the microvilli of PHA-E4 incubated cells were shortened significantly. These results indicate that lectins may induce direct effects on the intestinal epithelium.

The pathological effects of *P. vulgaris* isolectins on pig jejunal mucosa in organ culture are described in **chapter V**. The binding of PHA-E4 and PHA-L4 to the microvilli and microvillus vesicles in the top area of the villi was demonstrated by immuno-electron microscopy, whereas no binding could be demonstrated on the surface of the crypt cells. No differences were observed concerning the distribution of the isolectins.
In the explants cultured for five hours in the presence of the isolectins the enterocyte height and the villus length were decreased, and a lower villus/crypt ratio was calculated. At the ultrastructural level, the microvilli appeared to be shortened and irregularly positioned. Clusters of small vesicles, tied off from the microvilli, were seen in higher numbers when compared to control explants. These alterations were more severe in the PHA-E4 treated explants than in the PHA-L4 treated ones.
The activity of sucrase-isomaltase was decreased in PHA-E4 cultured explants, whereas the enzyme activity did not change in PHA-L4 incubated explants.
The alterations are probably caused by a direct effect of the isolectins on the villus epithelium.

A small intestinal mucosal biopsy technique in cannulated piglets is described in **chapter VI**. This technique enables a repeated sampling of small intestinal mucosa in the same animal. About 80 cm caudal to the ligament of Treitz a cannula was placed in the intestinal lumen. Through this cannula mucosal biopsies were gathered with a Laméris blind-biopsy tube for adults, nr 546. The mucosal biopsies were of good morphological quality. The biopsies may be used in the study of the interaction between administered noxious agents and the mucosal structure and function at consecutive times in the same animal. With this method, fewer animals are needed in an experimental study, since an experimental animal can also serve as its own control, before and after the experimental treatment. Moreover, the biopsies may be used for organ culture of small intestinal mucosa. Finally, this technique enables the sampling of biopsies in the Self emptying blind Pouch model.

The development of the Self-Emptying blind Pouch (SEP) model in the jejunum of piglets is described in **chapter VII**. This model was developed for the purpose of studying the effects of small amounts of potential enterotoxic substances on the structure and function of the small intestinal mucosa. Piglets were surgically provided with an one side closed Self-Emptying blind Pouch with intact blood vessel and nerve supply. At the blind end a cannula was fitted. Through this cannula mucosal biopsies were taken with a blind-biopsy tube. Biopsies were taken from 10 days post operatively onwards, twice a week during 6 weeks, and studied morphologically as well as functionally. During this period the jejunal mucosa in the pouch apart from a temporary postoperative decline in villus length and activity of sucrase-isomaltase and aminopeptidase, did not

change with regard to morphological as well as functional characteristics.

Chapter VIII deals with the effects of *P. vulgaris* isolectins E4 and L4 on the structure and function of the small intestinal mucosa in the Self-Emptying blind Pouch (SEP) model in piglets. Specific pathogen-free piglets were surgically provided with a SEP model in the mid-jejunum. At the blind end of the pouch a cannula was fitted. Through this cannula biopsies were taken beginning 10 days post operatively at regular intervals for 2½ weeks before and after the administration of *P. vulgaris* isolectins E4 and L4 through the cannula.

During the first 2½ experimental weeks the jejunal mucosa in the pouch did not change in morphological and functional characteristics, except for a temporary decline in villus length and activity of sucrase-isomaltase postoperatively.

Binding of the isolectins was confined to the villus epithelium. After administration of *P. vulgaris* isolectin-E4 the v/c ratio declined significantly. The glycocalyx was thickened and had a more fuzzy appearance, the microvilli were shortened and irregularly positioned, numerous microvillus vesicles were tied off. Forty-eight hours after the administration of PHA-E4 electron microscopically the effects were still visible, but were restored within 4 days. The activity of sucrase-isomaltase showed an initial decline but recovered rapidly. The effects of *P. vulgaris* isolectin-L4 were similar but less abundant.

SAMENVATTING

In dit proefschrift worden de effecten en de pathogenese van lectinen uit zaden van vlinderbloemigen, met name die van *Phaseolus vulgaris* (voerboon) op de structuur en de functie van darmepitheel cellen in verschillende hiertoe ontwikkelde *in vitro* en *in vivo* modellen beschreven.

In **hoofstuk II** wordt de literatuur handelend over de pathologische effecten van lectinen uit zaden van vlinderbloemigen op de structuur en de functie van het dunne-darmepitheel besproken. Na orale opname, kunnen biologisch actieve lectinen het lumen van de dunne darm bereiken. Hun binding aan het slijmvlies oppervlak kan een verhoogde aanmaak van mucinen en degeneratie van het darm-epitheel veroorzaken. De veranderingen van het epitheel van de dunne darm kunnen leiden tot het ontstaan van een hyperregeneratieve vlokatrofie en een verhoogd endogeen stikstof verlies, resulterend in diarrhee, een minder efficiente voeder conversie, verminderde groei, vermagering en de dood.

Hoofdstuk III behandelt de pathologische effecten van het voeren van rauwe *P. vulgaris* bonen op de structuur en de functie van het slijmvlies van de dunne darm bij het varken. Het slijmvlies werd gekarakteriseerd door een hyper-regeneratieve vlokatrofie. Vergeleken met de controle dieren, was het aantal slijmbekercellen ter hoogte van de vlokken significant verlaagd, terwijl dit in de crypten was verhoogd. De activiteit van de borstelzoom enzymen aminopeptidase en sucrase-isomaltase was significant lager in de met rauwe bonen gevoerde dieren.

Bovengenoemde bevindingen suggereren dat het voeren van *P. vulgaris* bonen aan biggen de absorptieve en digestieve capaciteit van de dunne darm reduceert, resulterend in diarrhee, vermagering en verminderde groei.

In **hoofdstuk IV** wordt de binding van de *P. vulgaris* isolectinen E4 en L4 aan gedifferentieerde humane colon carcinoma Caco-2 cellen en hun effect op het metabolisme en de microvillus structuur van deze cellen beschreven. Incubatie van de cellen met aan ferritine geconjugeerde isolectinen onthulde dat, gemeten aan het aantal gebonden ferritine deeltjes, PHA-E4 meer bond dan PHA-L4. Na 48 uur incubatie van de Caco-2 cellen met PHA-E4 of PHA-L4 werd de relatieve incorporatie van precursors voor DNA-, RNA-en (glyco)protein synthese in de trichloor-azijn-zuur onoplosbare fractie van de cellen bepaald. Beide isolectinen stimuleerden de inbouw van thymidine and glucosamine, terwijl geen van beiden in staat waren de inbouw van uridine te beinvloeden. Wat betreft de inbouw van fucose, methionine en N-acetyl-mannosamine bleef het stimulerende effect beperkt tot PHA-E4. Omdat PHA-E4 en L4 werden getest in dezelfde concentraties, blijkt dat PHA-E4 effectiever is in het opwekken van effecten dan PHA-L4. Vergeleken met controle en met

PHA-L4 geincubeerde cellen waren de microvilli van met PHA-E4 geincubeerde cellen significant verkort.
Deze bevindingen duiden pathogenetisch op een direct effect van lectinen op het darmepitheel.

In **Hoofdstuk V** worden de pathologische effecten van *P. vulgaris* isolectinen op het slijmvlies van het jejunum van biggen in orgaancultuur beschreven. M.b.v. immunoelectronen microscopie werd de binding van PHA-E4 en PHA-L4 aan de microvilli en de afgesnoerde microvillaire blaasjes aangetoond, terwijl er geen binding kon worden vastgesteld aan het oppervlak van crypte-epitheel. Er werd wat betreft de distributie van de isolectinen geen verschil gezien. In de biopten die gedurende 5 uur gekweekt werden in combinatie met de isolectinen waren de hoogte van de enterocyten en de lengte van de vlokken verkleind, de berekende vlok/crypte ratio was kleiner vergeleken met die in controle biopten. Op electronenmicroscopisch niveau bleken de microvilli verkort en onregelmatig geplaatst. In de behandelde biopten waren er in hogere mate clusters van microvillaire blaasjes afgesnoerd. De veranderingen waren het ernstigst in de met PHA-E4 behandelde biopten. De activiteit van het borstelzoom enzym sucrase-isomaltase was onder invloed van PHA-E4 gedaald, terwijl onder invloed van PHA-L4 er geen verandering was opgetreden. De beschreven veranderingen zijn waarschijnlijk veroorzaakt door een direct effect van de isolectinen op het vlokepitheel.

In **hoofdstuk VI** wordt een biopsie techniek in gecannuleerde biggen beschreven. Met behulp van deze techniek kunnen herhaaldelijk slijmvlies monsters van de dunne darm van hetzelfde dier worden genomen. Ongeveer 80 cm caudaal van het ligament van Treitz werd een cannule in het darmlumen aangebracht. Via deze cannule werden met een flexibele blinde biopteur (Laméris, nr 456) zuigbiopsiën van de dunne-darmmucosa genomen. De aldus verkregen biopsiën waren van goede morfologische kwaliteit. De biopsiën kunnen worden gebruikt in de studie naar de interactie van toegediende noxen en de structuur en functie van het slijmvlies in hetzelfde dier op opeenvolgende tijdstippen. Door deze techniek zijn er minder proefdieren nodig, omdat een dier fungeert als zijn eigen controle voorafgaand aan en na de experimentele behandeling. Verder kunnen de biopten ook worden gebruikt voor orgaan cultures van dunne-darmslijmvlies. Tenslotte kunnen met behulp van deze techniek slijmvliesbiopsiën genomen worden in het SEP model.

In **hoofdstuk VII** wordt de ontwikkeling van een zelfledigende blinde zak (Self Emptying blind Pouch model; SEP model) in het jejunum van het varken beschreven. Dit model werd ontwikkeld teneinde de pathologische effecten van kleine hoeveelheden potentieel toxische stoffen op de structuur en de functie van het dunne-darmslijmvlies te bestuderen. Hiertoe werden biggen operatief van een

zelfledigende blinde zak, met intacte bloedvat- en zenuwvoorziening, in het jejunum voorzien. Aan het blinde einde werd een cannule geplaatst. Via deze cannule kunnen slijmvliesmonsters met behulp van een zuigbiopteur worden verzameld. Vanaf 10 dagen post operatief werden er met regelmatige tussenpozen, gedurende 6 weken, slijmvliesmonsters uit de wand van de darmzak genomen. Deze biopsiën werden morfologisch en functioneel beoordeeld. Gedurende de experimentele periode bleek de structuur en de functie van het slijmvlies in de zak niet te zijn veranderd, behalve een tijdelijke post operatieve vermindering van de vloklengte en activiteit van sucrase-isomaltase en aminopeptidase.

In **hoofdstuk VIII** worden de effecten van *P. vulgaris* isolectinen op de structuur en functie van het slijmvlies van de dunne darm in het SEP model beschreven.

Hiertoe werden een aantal SPF biggen operatief van een SEP model in het jejunum voorzien. Via de cannule aan het blinde einde van de SEP werden vanaf 10 dagen postoperatief, met regelmatige tussenpozen gedurende 2 weken voor en na de toediening van de *P. vulgaris* isolectinen-E4 en L4 via de cannule slijmvliesbiopten verzameld. De morfologie van deze biopten werd zowel licht- als electronenmicroscopisch beoordeeld. Tevens werd biochemisch de activiteit van sucrase-isomaltase bepaald.

Gedurende de eerst twee en een halve experimentele weken voor de toediening van de isolectinen veranderde de structuur en de functie van het slijmvlies in het SEP model niet, behalve een tijdelijke postoperatieve vermindering van de vloklengte en de activiteit van sucrase-isomaltase.

De binding van de isolectinen was beperkt tot het vlokepitheel. Na de toediening van het *P. vulgaris* isolectine-E4 werd de lengte van de vlokken kleiner, werden de crypten dieper en daalde de v/c ratio significant. De glycocalyx was verdikt en pluiziger dan normaal, de microvilli waren verkort en onregelmatig geplaatst, grote hoeveelheden mivrovillaire blaasjes waren afgesnoerd. Achtenveertig uur na de toediening van PHA-E4 waren de effecten electronenmicroscopisch nog aanwezig, maar deze waren binnen de volgende 4 dagen hersteld. Dezelfde effecten traden in mindere mate op na de toediening van PHA-L4.

De activiteit van sucrase-isomaltase was in eerste instantie verminderd, maar herstelde snel.

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CURRICULUM VITAE

The author was born on the 24th of June 1956 in The Hague. After 5 years in Aruba, she returned to The Netherlands and attended the Rijks Scholen Gemeenschap in Brielle, passing the final examination in 1977. In 1986 the study Veterinary Medicine was finished. After some months as general practioner in a small animal clinic, work was started on April 1st, 1987 at the department of Veterinary Pathology of the Faculty of Veterinary Medicine.

De schrijfster van dit proefschrift werd geboren op 24 juni 1956 te 's-Gravenhage. Na een verblijf van 5 jaar op Aruba, werd bij terugkeer in Nederland aan de RSG te Brielle het VWO gevolgd. In 1986 werd het dierenarts diploma behaald. Na enige maanden werken in een kleine huisdieren praktijk, werd op 1 april 1987 gestart als toegevoegd onderzoeker bij de Vakgroep Veterinaire Pathologie van de Faculteit der Diergeneeskunde. In de periode van 1987 tot 1990 werd het onderzoek verricht.

geschenk Hr Krol '91