FACULTEIT DIERGENEESKUNDE

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TARGETING INTESTINAL INDUCTION SITES FOR ORAL IMMUNISATION OF PIGLETS AGAINST F4$^+$ *ESCHERICHIA COLI* INFECTION

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LIST OF ABBREVIATIONS

Ab	antibody
ASC	antibody-secreting cells
ADCC	antibody-dependent cell-mediated cytotoxicity
AEC	3-amino-9-ethylcarbazole
AEE	apical early endosome
APC	antigen presenting cell
APES	3-aminopropyl-triethoxysilane
ARE	apical recycling endosomes
ASC	antibody secreting cells
BCR	B cell receptor
BEE	basal early endosomes
BSA	bovine serum albumin
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CE	common endosomes
CD40L	CD40 ligand
CLSM	confocal laser scanning microscopy
CpGs	CpG oligodeoxynucleotides
CT	cholera toxin
CTL	cytotoxic T lymphocytes
Da	Dalton
DAB	diaminobenzidin
DABCO	1,4-diazobicyclo-(2,2,2)-octane
DC	dendritic cell
dpc	days post challenge
dpi	days post immunisation
E. coli	*Escherichia coli*
EGFR	epidermal growth factor receptor

ELISA	Enzyme-linked immunosorbent assay
ELIspot	Enzyme-linked immuno spot
ETEC	enterotoxigenic *Escherichia coli*
FAE	follicle-associated epithelium
Fc	fragment crystallisable of the antibody
FcαR	Fcα receptor
FITC	fluorescein isothiocyanate
F4R	F4 receptors
FLUOS	5(6)-carboxyfluorescein-N-hydroxysuccinimide ester
GALT	gut-associated lymphoid tissue
GC	germinal center
GI	gastrointestinal
GMS	glyceryl monostearate
HEV	high endothelial venule
HRP	horseradish peroxidase
IEL	intraepithelial lymphocyte
IFN-γ	interferon-γ
IFR	interfollicular region
Ig	immunoglobulin
IgR	immunoglobulin receptor
IgV genes	immunoglobulin variable genes
IL	interleukin
ID	intradermal(ly)
IM	intramuscular(ly)
i.p.	intraperitoneal
IPP	ileal Peyer's patches
J chain	joining chain
J_{mid}	mid-jejunum
JPP	jejunal Peyer's patches

J_{prox}	proximal jejunum
LE	late endosome
LP	lamina propria
LP_{mid}	lamina propria of the mid-jejunum
LP_{prox}	lamina propria of the proximal jejunum
LT	lymphotoxin or labile toxin of *Escherichia coli*
LTβR	lymphotoxin β receptor
Mab	monoclonal antibody
MADCAM1	mucosal vascular addressin cell adhesion molecule 1
MC	monomorphonuclear cells
MHC	major histocompatibility complex
MLN	mesenteric lymph node
MMC	migrating myoelectric complex
ND	not determined
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	peritoneal cavity
PE	polyethylene
pIgA	polymeric IgA
pIgR	polymeric immunoglobulin receptor
PMN	polymorphonuclear leukocytes
PMSF	phenylmethylsulfonyl fluoride
PP	Peyer's patch
RER	rough endoplasmatic reticulum
ROI	region of interest
RT	room temperature
SC	secretory component or subcutaneaous(ly)
SCID	severe combined immunodeficiency

SD	standard deviation
SDS	sodium dodecyl sulphate
SED	subepithelial dome
SEM	Standard error of the mean
sIgA	secretory IgA
SGF	simulated gastric fluid
SLC	secondary lymphoid organ chemokine
SPF	specific pathogen free
STa/b	stable toxin a/b
SWC	swine workshop cluster
TCR	T cell receptor
TEC	triethyl citrate
TECK	thymus expressed chemokine
TGF-β	transforming growth factor-β
TGN	trans-Golgi network
Th	T-helper
TJ	tight junction
Tr1	T regulatory type 1 cell
UEA	*Ulex europaeus*

PART I

INTRODUCTION

INTRODUCTION

Intestinal infections with enterotoxigenic *Escherichia coli* (ETEC) affect neonatal and recently weaned piglets. These infections cause diarrhoea and are responsible for severe economic loss due to growth retardation, elevated drug use and mortality. In general, most neonatal infections can be prevented by passive colostral and lactogenic immunity obtained by vaccination of the sow. However, this passive protection decreases with aging and disappears at weaning. As a consequence, the newly weaned piglet becomes highly susceptible to enteropathogens. In Belgium the losses due to postweaning ETEC diarrhoea are estimated to be approximately 13 000 000 euro/year. In order to protect the newly weaned piglet, an active immunity is needed in the form of antigen-specific secretory IgA (sIgA) in the gut lumen. However, available parenteral vaccines stimulate the systemic (IgG antibodies) rather than the mucosal immune system. Alternatively, oral vaccines should be used to stimulate the intestinal mucosal immune system.

Competent oral veterinary vaccines for inducing mucosal protection are not yet available. Indeed, the oral route of delivery is the most challenging and is difficult to exploit for proteins. The problems inherent with this route of delivery include: (i) low gastric pH and digestive enzymes causing degradation of the antigen; (ii) poor uptake of the antigen; and (iii) induction of oral tolerance instead of protective mucosal immunity by the gut-associated lymphoid tissue. Consequently, the antigen must not only survive the hostile gastric and intestinal intraluminal environments, it further has to interact with the intestinal epithelial cells and cross the epithelial barrier. However, interaction with the intestinal epithelial cells is hampered by the mucus gel layer, the cell surface glycocalyx and the closely packed microvilli, which not only act as a diffusion barrier, but also create a highly degradative microenvironment. Furthermore, to be effective as a vaccine, the antigen has to stimulate the intestinal immune system to produce a protective mucosal immunity. On the contrary, harmless antigens usually activate immunosuppressive mechanisms, resulting in oral tolerance.

Oral delivery systems can help to overcome these problems by reducing gastric and intestinal degradation of the antigen and by targeting the antigen to the specific immunological induction site(s) of the gut-associated lymphoid tissue. By doing so, these delivery systems reduce the dose of antigen needed to induce a protective immune response.

F4 (K88) fimbriae bearing ETEC ($F4^+$ETEC) are one of the most prevalent isotypes causing postweaning diarrhoea. In our laboratory it has been demonstrated that the oral administration of purified F4 fimbriae to weaned piglets can induce protection against subsequent challenge with $F4^+$ETEC. However, to induce a protective intestinal immune response at weaning, the piglet has to be vaccinated during the suckling period. The use of oral delivery systems which protect the F4 fimbriae both against gastric degradation as well as against F4-neutralising milk factors and antibodies, and which subsequently deliver the F4 fimbriae at the immunologic induction site(s) in the gastrointestinal tract will allow the most efficient vaccination. Furthermore, if mucosal adjuvants would be necessary, they also could be incorporated in the delivery system.

To develop oral vaccines, knowledge on the antigen transport across the intestinal epithelial barrier and on the mucosal immune system of the gut is necessary. Present knowledge on these two topics is reviewed in chapter 1 and 2, respectively.

CHAPTER 1

THE INTESTINAL EPITHELIAL BARRIER: ANTIGEN SAMPLING BY THE ENTEROCYTE AND THE M CELL A REVIEW

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1.1. INTRODUCTION

The primary function of the small intestine is to absorb nutrients (Thomson *et al.*, 2001). However, during the course of this nutrient absorption, the epithelium is exposed to a wide variety of antigens from food, resident bacteria, and invading micro-organisms. As a consequence, the intestinal epithelium has to be permeable for nutrients and macromolecules important for growth and development, such as for instance epidermal growth factor, whose size is comparable to that of many antigens (Weaver and Walker, 1988; Weaver *et al.*, 1990). Contrary, it also has to provide an effective barrier to potentially harmful macromolecules and micro-organisms. The mechanisms that have evolved to deal with this are extremely complex. On the one hand, the structural and functional properties of the epithelium limit the amount of antigen reaching the surface of the epithelium. On the other hand, the epithelium samples luminal antigens which are delivered to the cells of the mucosal immune system allowing a continuous immunosurveillance by which protection against harmful pathogens as well as tolerance to resident bacteria and harmless food antigens can be generated.

1.2. THE VILLOUS EPITHELIUM

The intestinal tract is lined by a simple epithelium, consisting of a monolayer of epithelial cells. These cells originate from multipotent stem cells present in the crypts (Loeffler *et al.*, 1993). The multipotent stem cells give rise to four major epithelial cells: 1) the absorptive enterocytes which make up >80% of all small intestinal epithelial cells (Cheng and Leblond, 1974); 2) the goblet cells which produce a variety of mucins (Falk *et al.*, 1994) and trefoil peptides needed for epithelial growth and repair; 3) the enteroendocrine cells which export peptide hormones (Roth *et al.*, 1990); 4) the paneth cells which secrete antimicrobial cryptdins or defensins, digestive enzymes, and growth factors (Bry *et al.*, 1994). Each small intestinal crypt supplies cells to several adjacent finger-shaped villi (Roth *et al.*, 1991; Gordon and Hermiston, 1994). Enterocytes, goblet and enteroendocrine cells migrate upwards to the villous tip during their differentiation (Cheng and Leblond, 1974; Hermiston *et al.*, 1993). Once on the villous tip, they enter a death program (Hall *et al.*, 1994) and are exfoliated from the villous' apically situated

extrusion zone into the intestinal lumen. The epithelium of the villus (derived from several surrounding crypts) is renewed every 3-4 days. On the contrary, paneth cells differentiate during a downward migration to the crypt base where they reside for an average of 23 days before being removed by phagocytosis (Cheng, 1974).

1.2.1. The mucus coat and cell-surface structures of the enterocytes

The uptake of macromolecules, particulate antigens and micro-organisms across intact epithelial monolayers is restricted by the mucus coat and luminal cell-surfaces structures of the enterocytes.

The mucus coat is composed of a solution of glycoproteins (mucin) of molecular weights ranging from one to several million dalton. Intestinal mucin molecules are made up of carbohydrate side chains (70 to 80%) bound to a protein skeleton. The exact composition of these molecules can vary greatly. Differences are found between animal species, within localized regions of the intestinal tract and during development (Cone, 1999; Corfield *et al.*, 2001). The mucus coat provides a filter overlying the surface of the epithelium. Indeed, the increased viscosity reduces the diffusion of molecules toward the epithelium (Strocchi and Levitt, 1991). This effect will be most marked for larger molecules, and therefore will limit the absorption of antigens rather than of nutrients, which are smaller. Furthermore, the carbohydrate moieties of the mucin molecules are analogous to the glycoprotein and –lipid receptors, present on the enterocyte membrane (Gibbons, 1981; Jin and Zhao, 2000). They can therefore act as competitors to the binding of proteins and micro-organisms on the enterocyte membrane. Moreover, the release of mucus into the lumen generates a stream that draws luminal contents away from the epithelium.

Enterocyte apical surfaces are covered by rigid, closely placed microvilli (Mooseker, 1985), the tips of which contain large, negatively charged, integral membrane mucin-like glycoproteins that form a continuous, filamentous brush border glycocalyx (Ito, 1974; Maury *et al.*, 1995). This thick (400-500 nm) layer contains adsorbed pancreatic enzymes and stalked intramembrane glycoprotein enzymes responsible for terminal digestion (Semenza, 1986). Furthermore, it serves as a diffusion barrier that

prevents direct contact of most macromolecular aggregates, particles, viruses, and bacteria with the microvillus membrane (Amerongen *et al.*, 1991; Apter *et al.*, 1993; Frey *et al.*, 1996). As a consequence the glycocalyx prevents the uptake of antigens and pathogens while providing a highly degradative microenvironment that promotes the digestion and absorption of nutrients.

The microvilli can also constitute a significant barrier because of their size and charge. In the intestinal epithelium of children (Phillips *et al.*, 1979), there are 40 microvilli of 100 nm diameter every 5 μm^2. If the microvilli move together, the distance between them can decrease until 25 nm, which is in the same order of magnitude of macromolecules; the dimension of albumin, for example, is 3 on 13 nm. Microvilli are negatively charged; consequently charged molecules may be significantly inhibited even if their diameter is well below 25 nm. The site of invagination of the plasma membrane is located in between the microvilli (Knutton *et al.*, 1974; Gonnella and Neutra, 1984). Thus, antigens have to pass the microvillus barrier to enter the enterocyte. This has a direct relevance to disease processes, as any agent that causes microvillus atrophy or affects the formation of the microvilli will alter the barrier function of the intestine.

1.2.2 Macromolecular transport through the enterocyte

Several reports demonstrate the transport of macromolecules from the lumen of the GI tract across the villous epithelium. Macromolecules can be transported through the enterocyte (transcellular transport) or between adjacent epithelial cells (paracellular transport).

1.2.2.1. Transcellular transport

The plasma membrane of the enterocyte is composed of a lipid bilayer in which membrane-bound proteins and glycoproteins are situated. Because of its physical structure, it is very unlikely that macromolecules can cross the lipid bilayer into the enterocyte cytosol. Therefore, most macromolecules are only efficiently transported into the enterocyte by receptor-mediated endocytosis. Consequently, membrane binding is important in transporting macromolecules across the cell (Stern and Walker, 1984). Binding to the surface of the cell depends on the antigen structure (Stern and Walker,

1984) and also on the chemical composition of the microvillous membrane. This is important since for example the lipid composition of the plasma membrane changes with development (Pang *et al.*, 1983; Chu and Walker, 1988).

1.2.2.1.1. Different mechanisms of endocytosis

Nutrient molecules, such as sugars, amino acids and ions, enter the intestinal cell cytoplasm at the apical membrane by *epithelial transporters* (integral membrane protein pumps or channels) and exit the basolateral membrane. In contrast to these small molecules, *macromolecules* enter the cell in *membrane-bound vesicles that derive from invagination and pinching-off of pieces of the apical membrane* in a process termed endocytosis. Besides uptake of macromolecules, endocytic mechanisms are also involved in regulation of cell-surface receptor expression, maintenance of cell polarity, and antigen presentation (Conner and Schmid, 2003). The endocytosis by the enterocyte is restricted to pinocytosis, as enterocytes can not perform phagocytosis which is primarily conducted by specialised cells, including macrophages, monocytes and neutrophils (Aderem and Underhill, 1999). *Four different basic mechanisms of pinocytosis* exist, namely macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis of which macropinocytosis is unlikely to occur by enterocytes since it involves extensive membrane ruffling and sampling of large volumes of the extracellular milieu (Conner and Schmid, 2003).

Clathrin-mediated endocytosis is the major endocytic pathway. It involves the concentration of high-affinity transmembrane receptors and their bound ligands into 'clathrin-coated pits' on the plasma membrane. These coated pits are formed by the assembly of cytosolic coat proteins, of which the major unit is clathrin, on the cytosolic surface of the plasma membrane (Knutton *et al.*, 1974; Gonnella and Neutra, 1984; Conner and Schmid, 2003). This clathrin assembly requires assembly proteins which in turn interact with adaptor proteins. The adaptor proteins specify the site of clathrin assembly and select the material for transportation into the cell by interacting with the internalisation motives - endocytic sorting determinants - located within the cytosolic domain of the receptors (Bonifacino and Dell'Angelica, 1999; Mishra *et al.*, 2002). The life

time of clathrin-coated pits is short; within about a minute of being formed, they invaginate and pinch off to form endocytic vesicles that are encapsulated by a clathrin coat and carry concentrated receptor-ligand complexes into the cell. The macromolecules enter the vesicle bound to the surrounding membrane by their own receptor (receptor-mediated pinocytosis) or by non-specific attraction (adsorptive pinocytosis); they can also enter the vesicle free in solution (Sanderson and Walker, 1993). The clathrin-coated vesicles are even more transient than the coated pits; within seconds of being formed, they shed their coat and are able to fuse with early endosomes (Robinson, 1987; Brodsky, 1988).

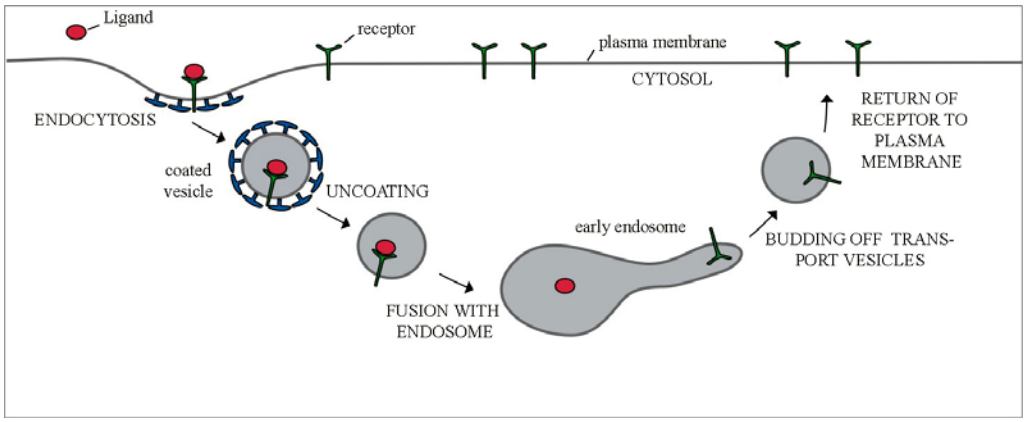

Fig. 1.1. Clathrin-mediated endocytosis.
High-affinity receptors and their bound ligands are concentrated into a clathrin-coated pit, after which a clathrin-coated vesicle is formed. Following uncoating, the vesicle can fuse with an early endosome. In the present example, the ligand dissociates from its receptor in the more acidic environment of the endosome, after which the receptor returns to the plasma membrane. For simplicity, only one receptor is shown entering the cell and returning to the plasma membrane.

Caveolae are flask-shaped invaginations of the plasma membrane that demarcate cholesterol and sphingolipid-rich microdomains, in which many diverse signaling molecules and membrane transporters are concentrated (Anderson, 1998). The shape and structural organization of caveolae are determined by caveolin, a dimeric protein that binds cholesterol, after which it inserts into the plasma membrane and self-associates to form a caveolin coat on the surface of the membrane invaginations. The caveolae are static structures (Thomsen *et al.*, 2002), which internalization can be triggered by binding to receptors in the caveolae (Nabi and Le, 2003). However, even after activation the

caveolae are only slowly internalized (t1/2>20 min) and form small vesicles (50-60 nm in diameter) that carry little fluid-phase volume. Consequently, it is unlikely that this process contributes significantly to bulk fluid-phase uptake (Conner and Schmid, 2003).

Like caveolae, other microdomains of highly ordered (glyco)sphingolipids and cholesterol exist in the plasma membrane and are generally referred to as lipid *rafts*; small structures, 40-50 nm in diameter, that diffuse freely on the cell surface (Edidin, 2001). The small rafts can presumably be captured by, and internalized within any endocytic vesicle. For example both Shiga toxin and non-aggregated cholera toxins, which bind to raft-associated glycolipids, are internalized by clathrin-coated vesicles (Sandvig *et al.*, 1989; Thomsen *et al.*, 2002). On the other hand, the rafts can invaginate and bud off in a caveolin- and clathrin- independent way. The mechanisms that regulate these caveolin- and clathrin-independent endocytosis are still poorly understood. Ligand binding may serve not only to recruit receptors to rafts but also contribute to the formation and internalization of these domains (Nabi and Le, 2003).

1.2.2.1.2. Endocytosis in the polarized enterocyte

The enterocytes are highly polarized cells with biochemically and functionally distinct plasma membrane domains. This polarization is established and maintained by homotypic interactions of adhesion molecules such as E-cadherin (Drubin and Nelson, 1996), heterotypic interactions of integrins with extracellular matrix components (Hynes, 1992) and polarised cytoskeletal and signaling networks (Drubin and Nelson, 1996). The tight junctions, that seal the apical poles of the cells, prevent lateral diffusion of glycolipids and proteins between apical and basolateral domains of the plasma membrane. The basolateral surface comprises a lateral subdomain involved in cell-cell adhesion using adhesion molecules, adherens junctions, desmosomes, gap junctions and interdigitations, and basal subdomain interacting with the extracellular matrix and basement membrane (Kato and Owen, 1999).

The polarized distribution of newly synthesized plasma membrane proteins results from their selective delivery to and retention at the appropriate membrane (Mostov *et al.*, 2000; Matter, 2000) (Fig. 1.2.). In the case of direct delivery, the sorting site for apical and

basolateral membrane proteins is the trans-Golgi network (TGN) where these proteins are incorporated into apical or basolateral vesicles that are targeted to the respective surfaces (Matter and Mellman, 1994). In the indirect route, the membrane proteins are transported to one of both surfaces, usually the basolateral surface. From there, they are endocytosed and sorted to the correct surface domain, where they are stabilized. The sorting site in the indirect pathway remains to be determined, but is likely that the relevant sorting event occurs in the endosomes after endocytosis (Fig. 1.1). Selective targeting requires that the membrane proteins carry sorting determinants recognized by a specific sorting machinery in the TGN and endosomes. Apical targeting from the TGN or endosomes has been attributed to a number of different types of signals, including N-linked or O-linked carbohydrates, signals located in the extracellular, transmembrane and cytoplasmic domains, as well as in the lipid anchor of glycosylphosphatidylinositol anchored proteins. Targeting determinants that specific basolateral sorting in the TGN and endosomes are amino-acid sequences in the cytoplasmic domains of the membrane proteins, related or unrelated to clathrin-coated pit signals (Mostov et al., 2000; Matter, 2000).

After the endocytic uptake of either adsorbed or fluid-phase macromolecules via clathrin, caveolin- or non-coated vesicles and pits, these vesicles fuse to form an early endosome. In the polarised enterocytes, distinct sets of apical and basolateral early endosomes exit that differ in function and composition. Apical and basolateral early endosomes cannot fuse with each other, but both fuse with common endosomes and late endosomes (Apodaca, 2001). In the early endosome, certain proteases are delivered (Courtoy, 1991) and the lumen is acidified to pH 6.0 to 6.2, a milieu in which certain ligands are released from their receptors (Maxfield and Yamashiro, 1991). Membrane proteins, lipids, and content may be sorted in the early endosome for rapid return to the same cells surface (recycling, whether or not via the apical recycling endosome (ARE)), for transport along the degrative pathway (to the late endosome and ultimately to lysosomes), or for transport to the opposite membrane domain (transcytosis, whether or not via the common endosome) (Mostov *et al.*, 2000; Apodaca, 2001) (Fig. 1.2.).

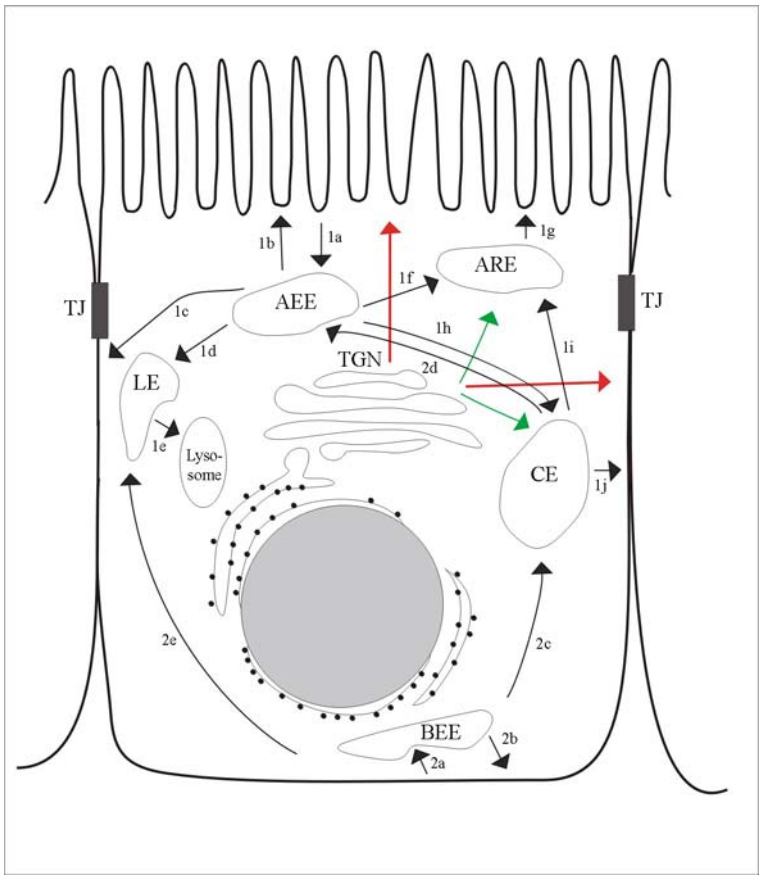

Fig. 1.2. Model for endocytic traffic in polarized epithelial cells.
The apical and basolateral surfaces are separated by tight junctions (TJ). Upon internalization, fluid and membrane components are delivered to distinct early endosomes, the apical early endosome (AEE) (1a) or the basal early endosome (BEE) (2a). From the AEE, internalized components can recycle (1b) or transcytose (1c), can be delivered to late endosomes (LE) (1d) and ultimately lysosomes (1e). They can also be delivered to the apical recycling endosome (ARE) (1f) for recycling (1g) or to the common endosomes (CE) (1h) for recycling through the ARE (1i) or to transytose (1j). From the BEE, internalised components can recycle (2b), be delivered to the CE (2c) for recycling to the basolateral membrane (1j) or transcytosis through AEE (2d/1b) or ARE (1i/1g) or they can be delivered to the LE (2e) and lysosomes (1e).
Newly synthesized membrane proteins can directly be delivered to the apical or basolateral surface after sorting in the trans-Golgi network (TGN) (red lines). However, evidence exists that some of the traffic from the TGN to one or both surfaces intersects with material endocytosed form either surface. The location(s) of these meeting point(s) is(are) not known, but might include the CE and/or ARE (green lines) (Mostov et al., 2000). In the indirect route, the proteins are transported to one of both surfaces, followed by endocytosis, transcytosis and stabilization of the protein at the correct surface domain by interactions with the submembrane cytoskeleton.

After uptake of the macromolecules, most of them will be destroyed by enzymes present in the lysosome or in the endocytic vesicle (Dinsdale and Healy, 1982). However, some of them escape degradation and are released into the interstitial space, after which they enter the systemic circulation with or without prior absorption into the lymphatic vessels (O' Hagan *et al.*, 1988). Membrane-bound molecules are more likely to transverse

the cell than soluble molecules (Sanderson and Walker, 1993). Quite large particles seem endocytosable by the enterocytes since particles with a size of 200 nm up to 2 μm have been reported to be taken up in intracellular vesicles by enterocytes, to be released into the lamina propria and subsequently translocated to the mesenteric lymph nodes within 5 to 30 min after oral administration (Sanders and Ashworth, 1961; Hodges *et al.*, 1995; Hazzard *et al.*, 1996). However, the transport of particles smaller than 50 nm seems to be preferable (Ponchel and Irache, 1998).

1.2.2.2. Paracellular transport

The cells of the intestinal epithelium are joined together by tight junctions. These tight junctions make paracellular transport of large molecules impossible. However, the tight junction is a dynamic structure and events taking place in the epithelium, the lamina propria and the intestinal lumen may affect its resistence.

Transient and reversible increases in tight junction permeability to luminal peptides occur naturally as a consequence of activation of certain apical membrane transport systems. Na^+-coupled transport of glucose and amino acids induces the condensation of microfilaments in the zone of the perijunctional actomyosin ring, thereby dilating the tight junctions and enhancing the absorption of nutrients by solvent drag due to a transjunctional osmotic flow (Pappenheimer and Volpp, 1992). The open tight junction has a pore radius of 5 nm and allows the passage of small macromolecules (4000 to 5500 Da) (Pappenheimer *et al.*, 1994; Pappenheimer, 1988; 2001). A polypeptide of 11 amino acids long (1900 Da), but not larger immunogenic proteins (horseradish peroxidase, 40 kDa), has been shown to pass through this route after glucose-elicited dilatations (Atisook and Madara, 1991). However, Zhang and Castro (1992) suggested that the uptake of larger macromolecules (ovalbumin (45 kDa) and *Thrichinella spiralis* antigen) might also occur via this paracellular route and demonstrated that the enhanced permeability of the paracellular pathway following activation of an apical glucose transporter can successfully enhance immune responsiveness to specific luminal antigens (i.e. enhanced boosting of the immune system) by providing greater access to the mucosal immune system (Zhang and Castro, 1992).

On the other hand, pathologic insults to the intestine may open these pores sufficiently to allow passage of larger antigens. Microbial interactions with the apical membrane of the enterocytes can cause rearrangements of F-actin and of associated proteins via phosphorylation of critical enzymes leading to increased permeability of tight junctions (Yuhan *et al.*, 1997; Philpott *et al.*, 1998). The zonula occludens toxin, a protein elaborated by *Vibrio cholerae*, induces modifications of the cytoskeletal organization leading to the opening of the tight junctions and to enhanced intestinal absorption of orally administered large macromolecules such as IgG (140-160 kDa) (Fasano *et al.*, 1997; Fasano and Uzzau, 1997). Cholera toxin also opens the tight junctions (Holmgren *et al.*, 2003). However, this effect is probably indirect and caused by the CT-induced cytokines. Indeed cytokines can affect the epithelial permeability; both IFN-γ, TNF-α, IL-1β, IL-4 as IL-13 act on epithelial receptors to increase the permeability of tight junctions, whereas TGF-β enhances the epithelial barrier function (Madara and Stafford, 1989; Berin *et al.*, 1999; McKay and Baird, 1999 Matysiak-Budnik *et al.*, 2001). IL-10 may downregulate epithelial permeability by reducing the production of T cell proinflammatory cytokines or possibly by acting directly on the epithelium (McKay and Baird, 1999). The epithelial permeability can further be changed by growth factors; fibroblast growth factor and epidermal growth factor (McKay and Baird, 1999; Chen *et al.*, 2001; Banan *et al.*, 2003) enhance the barrier properties of the epithelium, whereas insulin and insulin-like growth factors increase the paracellular permeability (McRoberts and Riley, 1994). Moreover, the barrier properties of the epithelium are influenced by the enteric nervous system, with cholinergic neurotransmittors clearly facilitating enhanced passage of macromolecules through the tight junctions (Bijlsma *et al.*, 1996).

1.2.3. Enterocyte as antigen presenting cell

It has been speculated that enterocytes function as antigen presenting cells (APC) and can regulate T cell responses in the intestinal mucosa (Hershberg and Mayer, 2000). The enterocytes contact T cells within the epithelium, namely the intra-epithelial lymphocytes, and T cells in the underlying lamina propria via basolateral projections through the semi-porous basement membrane. In order to act as APC, the enterocyte

must be able to internalise and process antigen, which is shown in several studies (Gonnella and Wilmore, 1993; Srobel and Mowat, 1998; Hershberg *et al.*, 1998).

1.2.3.1. Interaction with CD4±T cells.

Enterocytes from human, rat and mouse constitutively express MHC class II molecules, with enhanced expression in states of inflammation (Kaiserlian *et al.*, 1989; Mayer *et al.*, 1991; Bland and Whiting, 1992). Furthermore, they are able to process and present antigen via their MHC class II molecules (Kaiserlian *et al.*, 1989; Brandeis *et al.*, 1994; Hershberg *et al.*, 1997). The expression of the MHC class II molecules is mostly restricted to the basolateral membrane, where the cell contacts the intra-epithelial and lamina propria lymphocytes (Mayrhofer and Spargo, 1990; Hershberg *et al.*, 1998). Endocytosis in the polarized epithelial cells from the apical surface differs from uptake from the basolateral face (Gottlieb *et al.*, 1993; Jackman *et al.*, 1994). Antigen trafficking from the respective polarized surfaces possibly allows selective exposure to specific proteases en route to a class II loading compartment, as it affects the functional outcome with regards to the generation of T cell epitopes (Hershberg *et al.*, 1998). The polarized expression of various surface receptors on the enterocytes may modulate the antigen processing via class II pathways by enhancing the uptake of specific antigens and/or by targeting these antigens to certain intracellular compartments. The processing of luminal antigens normally exposed only to the apical surface might have a different immunologic outcome when these antigens gain access to the basolateral surface of the enterocytes via leaky tight junctions. An antigen which normally elicits no significant responses or a tolerogenic response when processed apically, may become immunogenic after processing from the basolateral membrane due to the different processing (Hershberg and Mayer, 2000).

In the absence of inflammation, the enterocytes do not express the costimulatory molecules CD80 (B7-1) or CD86 (B7-2) (Sanderson *et al.*, 1993; Bloom *et al.*, 1995). Since antigen presentation in the absence of these costimularory molecules results in the induction of anergy and thus tolerance, the epithelial cell presentation may be involved in down-regulating T cell responses under normal conditions. Subsequently, the

enterocyte can actively inhibit the immune response initiated by the passage of antigen that has leaked through to professional APC in the lamina propria. However it is not unreasonable to suggest that under pathologic conditions the enterocytes function as professional APC and stimulate mucosal $CD4^+$ T cell responses. *H. pylori*, for example, induces expression of costimulatory molecules on gastric epithelium (Ye *et al.*, 1997). Furthermore, human enterocytes constitutively express CD58 (LFA-3) and T cells of the lamina propria display an enhanced signaling via CD2, the ligand for CD58, resulting in vigorous proliferation and cytokine release (Qiao *et al.*, 1991; Targan *et al.*, 1995). This contrasts with peripheral blood T cells which are predominantly activated in an antigen-specific way via the TCR/CD3 complex. As a consequence, the signaling via CD58 on enterocytes may be relevant for these lamina propria T cells in the intestinal mucosa (Framson *et al.*, 1999).

Porcine enterocytes do not express MHC class II molecules making their role as antigen presenting cells very unlikely (Stokes *et al.*, 1996). However, in the lamina propria, a huge amount of cells express the MHC class II molecules, including classical antigen presenting cells (mainly DCs) as well as non-professional APC of which the eosinophils predominate (Stokes *et al.*, 1996; Haverson *et al.*, 2000). The capillary endothelial cells have also been shown to be MHC class II positive, which indicates the potential for interaction with lamina propria $CD4^+$T cells. Electron microscopic studies demonstrated the close association between lymphocytes in the capillary lumen and the luminal surface of the endothelial cells, which might suggest that the flow of lymphocytes may be selectively delayed by this MHC class II - CD4-CD3/TCR interaction and thus may play a role in lymphocyte recirculation (Wilson *et al.*, 1996; Stokes *et al.*, 1996). However, the ability of these endothelial cells to present antigen to $CD4^+$T cells as a non-professional APC may not be ruled out. Such presentation of antigens (transported through the enterocyte) in absence of costimulatory signals will result in anergy of the $CD4^+$ T cells.

1.2.3.2. Interaction with $CD8^+$T cells

Although epithelial cell lines have been shown to be good targets for class I-restricted virus-specific cytotoxic T lymphocytes (CTL), they fail to prime an antiviral CTL response (Hershberg and Mayer, 2000). However, the enterocytes may induce $CD8^+$T cell responses, since human and mouse enterocytes have been shown to express class Ib molecules (Bleicher *et al.*, 1990; Blumberg, 1998). Human enterocytes express, for example, CD1d, MICA and MICB (Groh *et al.*, 1998). The ligands presented by these unusual class I molecules are non-peptide antigens, like components of the bacterial cell wall and lipids (Joyce *et al.*, 1998; Braud *et al.*, 1999). However, it has been demonstrated that mouse CD1 can bind relatively long hydrophobic peptides in its hydrophobic antigen-binding site, although this binding is less easy than lipid binding (Brossay et al., 1998).

The CD1d molecule associates with gp180, a novel CD8 ligand, and activates a subpopulation of $CD8^+$ regulatory cells whose function is to suppress the immune response in an antigen non-specific way (Mayer, 1998; Campbell *et al.*, 1999). Via MICA and MICB, intestinal T cells bearing the γδ-TCR can be stimulated (Groh *et al.*, 1998). Although CD1d and MICA/MICB may induce T cell responses, a functional role of these class Ib molecules *in vivo* under physiological or pathological conditions in the gut mucosa has not been demonstrated.

1.2.3. Antigen sampling by dendritic cells

Rescigno *et al.* (2001a) demonstrated that dendritic cells (DC) can take up bacteria directly from the intestinal lumen without disturbing the integrity of the epithelial barrier. Hereto, the DCs open the tight junctions between the epithelial cells, establish tight-junction-like structures with the epithelial cells and send dendrites outside the epithelium. It is hypothesized that this newly identified mechanism of immunosurveillance could play a dominant role in the adaptive immunity considering the abundance of DCs in the subepithelial dome (SED), beneath the follicle-associated epithelium, and in the lamina propria, as well as their ability to deliver antigens into lymphoid tissues where an efficient immune response can be generated (Gewirtz and

Madara, 2001). DCs may deliver luminal antigens to the draining mesenteric lymph nodes (MLNs) (Pron *et al.*, 2001) which play an important role in the induction of mucosal and systemic Ab responses after oral immunisation (Yamamoto *et al.*, 2000). Interestingly, Rescigno *et al.* (2001a) found that DCs rapidly leave the epithelium after sampling of the pathogen *Salmonella typhimurium*, whereas they remain in place after their interaction with a commensal *Eschericha coli*, demonstrating that the DC was able to discriminate between pathogens and commensals. However the importance of this type of antigen sampling in mucosal immunity and/or tolerance still has to be determined.

Although most intestinal epithelial cells are shed into the intestinal lumen following apoptosis at the villus tip, some are endocytosed by a subpopulation of DCs in the LP and PP. These DCs constitutively transport such apoptotic intestinal epithelial cells to the T cell areas of MLNs (Huang *et al.*, 2000). There, the acquired self-antigens in the form of apoptotic epithelial cells may be presented by the DCs to tolerize naive T cells. Alternatively, a protective immune response may be stimulated against pathogens that primarily infect the epithelial cell or against antigens that have been endocytosed. Since the uptake of apoptotic cells in the absence of inflammation will not induce maturation of the DCs, it is hypothesized that these immature DCs induce tolerance to self-antigens within phagocytosed apoptotic bodies derived from the normal turnover of tissues (Steinman et al., 2000). This will occur well before the entry of a foreign antigen, so when infection and DC maturation take place, the immune system can focus on the foreign peptides that the DCs have processed.

1.3. THE FOLLICLE-ASSOCIATED EPITHELIUM

To allow a continuous immunosurveillance of the intestine, antigens have to be transported through the epithelial barrier. In contrast to the villous epithelium, where the transport of macromolecules is variable, macromolecules are transported in a controlled manner by specialised epithelial cells, M cells, present in the follicle-associated epithelium (FAE), overlying the B cell follicles of the Peyer's patches (Bockman and Cooper, 1973; Owen, 1977).

1.3.1. Morphology

1.3.1.1. Morphological features of the FAE

The FAE differs in cell composition from villous epithelium: enteroendocrine cells and mucus-producing goblet cells are absent or rare (Owen, 1977; Gebert and Cetin, 1998), whereas distinct follicle-associated enterocytes and the characteristic M cells are present. Furthermore, a lower number of Paneth cells are present in the follicle-associated crypts (Giannasca *et al.*, 1994).

The follicle-associated enterocytes differ from villous enterocytes. They are also coated with a tick filamentous brush border glycocalyx (Maury *et al.*, 1995; Frey *et al.*, 1996), but express lower amounts of digestive enzymes (Owen and Bhalla, 1983; Savidge and Smith, 1995). Moreover, the FAE is covered with less mucus (Owen, 1999), due to the absence or rarity of goblet cells. In addition, the entire FAE is devoid of polymeric Ig receptors, consequently no protective secretory IgA (sIgA) is transported from the interstitium towards the lumen (Pappo and Owen, 1988). These characteristics promote local contact of intact antigens and macromolecules with the FAE. The FAE further lacks the subepithelial myofibroblasts that form a sheath under the epithelium of villi, and the basement membrane differs from that of the villi: it lacks laminin-2 and perlecan and is highly porous, containing holes that presumably reflect the frequent migration of cells into and out of the epithelium (McClugage *et al.*, 1986).

1.3.1.2. Morphological features of the M cell

The term ‘M cell’ was introduced by Owen and Jones (1974) as the ‘microfold cell’, referring to the shape of the luminal surface projections of the human M cells, namely small microfolds. In mice, however, M cells have small irregular microvilli rather than microfolds, therefore the abbreviation M cell was later used to designate ‘membranous epithelial cell’ (Owen, 1977). The term refers to the M cell cytoplasm that forms a membrane-like, thin apical bridge separating the intestinal lumen from the subepithelial space.

M cells are attached to adjacent cells in the FAE by tight junctions, desmosomes and interdigitations. Their luminal surface is characterized by the absence of overlying

mucus. The brush border glycocalyx is poorly developed (Frey *et al.*, 1996). In contrast to the villous enterocyte, the expression of the brush border digestive enzymes is often, but not always, reduced or absent (Owen and Bhalla, 1983; Savidge and Smith, 1995; Sierro *et al.*, 2000). This indicates that digestion and absorption of luminal contents are probably not the major functions of these M cells. M cell microvilli are spars, irregular in size, shape and arrangement, and lack an organised terminal web of microfilaments. The large intermicrovillar endocytic domains (Neutra *et al.*, 1987), the frequent apical pits, tubulovesicular structures and an abundance of cytoplasmic vesicles indicate an involvement in cellular transport (Neutra *et al.*, 1988; Ermark *et al.*, 1995). The basolateral membrane is invaginated to form an intraepithelial pocket in which T lymphocytes (mostly $CD4^+$ helper cells and CD45RO memory cells), B lymphocytes (naïve $sIgD^+$ and memory $sIgD^-$ B cells) and antigen presenting cells, and occasionally plasma cells and polymorphonuclear leukocytes (PMNs) are present (Wolf and Bye, 1984; Neutra *et al.*, 1996a). Neutra *et al.* (2001) think that a lectin-like receptor is involved in the lymphocyte homing into the M cell pockets, as the basolateral membrane expresses oligosaccharide epitopes not expressed by neighboring enterocytes (Giannasca *et al.*, 1994).

The proportion of M cells in the FAE ranges form 10% in humans and rodents, to 50% in rabbits and pigs and 100% in the terminal ileum of calves (Owen and Ermak, 1990; Wolf and Bye, 1984; Clark *et al.*, 1993; Gebert *et al.*, 1994).

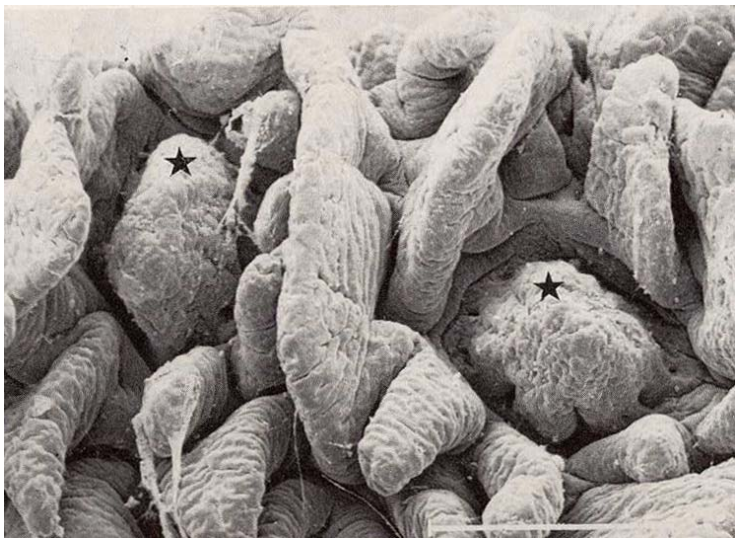

Fig. 1.3. Scanning electron micrograph of two dome-shaped lymphoid nodules (asterisks) surrounded by finger-shaped villi inside the area of the Peyer's patch in the ileum of a three week-old piglet (bar = 400 μm). Reproduced with permission from Vellenga *et al.*,1985.

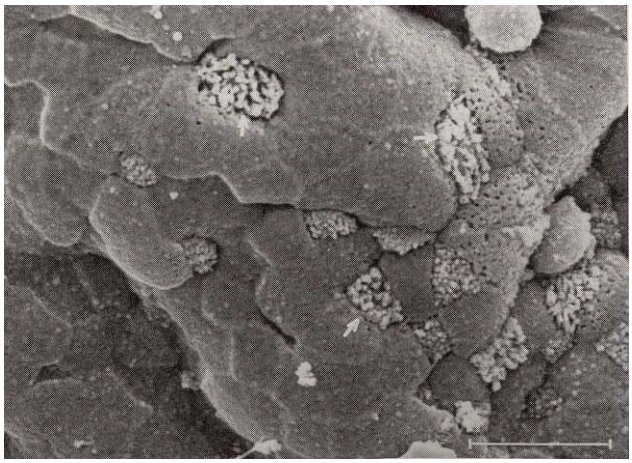

Fig. 1.4. Scanning electron micrograph of a dome-shaped lymphoid nodule inside the area of the Peyer's patch in the ileum of a three week-old piglet. Some typical M cells (arrows) are visible (bar = 10 μm). Reproduced with permission from Vellenga *et al.*, 1985.

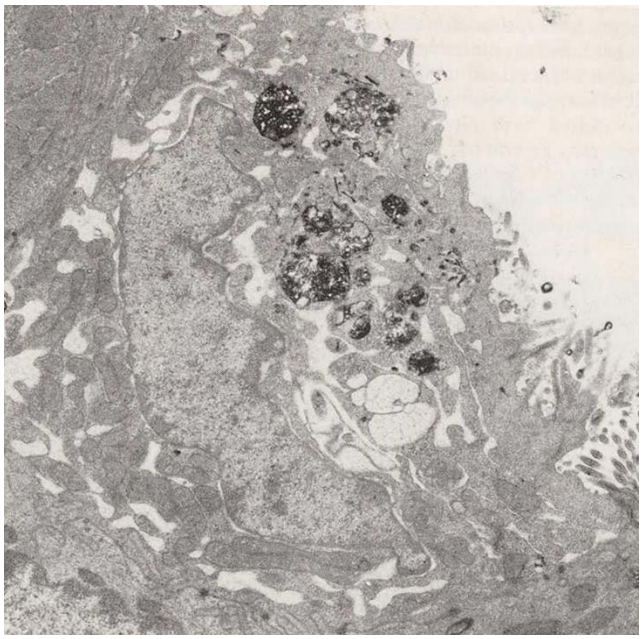

Fig. 1.5. Transmission electron micrograph showing a porcine M cell with vesicles containing horseradish peroxidase (black deposits) after intraluminal exposure during one hour. Reproduced with permission from Vellenga *et al.*, 1985.

1.3.1.3. M cell apical membrane glycoconjugates and proteins

Although M cells lack the uniform thick glycocalyx, the apical membrane does express abundant glycoconjugates in a cell coat that varies widely in thickness and density (Bye *et al.*, 1984; Neutra *et al.*, 1987). However, this M cell glycosylation pattern is distinct from those of enterocytes, which is reflected by the occurrence of characteristic lectin-binding sites on the M cell surface (Clark *et al.*, 1993; Giannasca *et al.*, 1994; Lelouard *et al.*, 1999, 2001a). Furthermore, the M cell population itself is heterogeneous as is reflected by the glycosylation patterns. These patterns vary within a single FAE along the crypt-dome axis, possibly reflecting different stages of cell differentiation (Gebert and Posselt, 1997) and vary between different radial cell strips that converge at the top of the dome (Gebert and Posselt, 1997). They can further vary between different FAE in distinct sections of the gut and between different species (Gebert and Hach, 1993; Giannasca *et al.*, 1994; Clark *et al.*, 1994a). For example the *Ulex europaeus* (UEA) I lectin, recognizing several carbohydrate structures containing α-(1-2)fucose, stains selectively M cells in the FAE of Peyer's patches of BALB/c mice (Clark *et al.*, 1993; Falk *et al.*, 1994), whereas it does not bind to human M cells but does strongly bind to all human enterocytes (Giannasca *et al.*, 1999). Contrary, human M cells have been shown to

preferentially display the sialyl Lewis A antigen (Neu5Acα(2-3)Galβ(1-3)GlcNAc[Fucα(1-4)]) (Giannasca *et al.*, 1999). However, the specificity of sialyl Lewis A for human M cell could not be confirmed by Wong and colleagues (2003). This M cell diversity might expand the ability of these antigen-sampling cells to interact with and deliver a wider variety of luminal antigens to the underlying antigen-processing cells (Giannasca *et al.*, 1994; Neutra *et al.*, 1996a). This diversity may determine the tropism of pathogens that exploit M cells for invasion (Clark *et al.*, 1994a, 1994b).

Membrane proteins specific to M-cell apical surfaces have not been identified, except for β1-integrin, a protein that is located basolateral on other epithelial cells. This protein may be exploited by pathogenic *Yersinia* in order to attach and invade the M cells (Schulte *et al.*, 2000).

1.3.1.4. M cell intermediate filaments

The unusual shape of the M cell appears to be maintained by a dense network of intermediate filaments, which forms an arch around the pocket and a thick network around the nucleus (Neutra *et al.*, 1988). The cytoskeleton of rabbit M cells is unusual for epithelial cells in that it contains vimentin (Gebert *et al.*, 1992; Jepson *et al.*, 1992) and specific cytokeratins (Gebert *et al.*, 1992; Jepson *et al.*, 1992; Gebert *et al.*, 1994; Rautenberg *et al.*, 1996). Rat M cells strongly express cytokeratin 8 (Rautenberg *et al.*, 1996), whereas porcine M cells strongly express cytokeratin 18 (Gebert *et al.*, 1994). In humans, the composition of the intermediate filaments does not differ from that in enterocytes (Kucharzik *et al.*, 1998), whereas in mice, the actin-bundling protein villin, concentrated in the microvilli of enterocytes, is diffusely distributed in the cytosol (Kernéis *et al.*, 1996), reflecting the modified apical organization and perhaps the ability of the M cell to rapidly respond to adherence of micro-organisms with ruffling and phagocytosis.

1.3.2. Ontogeny of the FAE and M cells

M cells are not randomly distributed over the dome epithelium; they are arranged in radial strips that converge at the top of the dome and originate from specialized dome-associated crypts. These M cell rich strips alternate with strips poor or devoid of M cells

that are associated with ordinary crypts lying more peripheral to the domes (Gebert *et al.*, 1999). The dome-associated crypts differ from the ordinary crypts in size, shape and cellular composition. Cells originating from these crypts can migrate/differentiate differently depending on their localization (Gebert *et al.*, 1999). Cells at the villous side differentiate into absorptive enterocytes, goblet cells and enteroendocrine cells that migrate onto the villi; these cells acquire secretory functions and express receptors for polymeric immunoglobulin. Contrary, cells at the dome side fail to express these receptors and move onto the dome where they differentiate into M cells and distinct follicle-associated enterocytes (Pappo and Owen, 1988).

M cell differentiation is probably induced by cell contacts and/or diffusible factors from the underlying lymphoid follicles and/or micro-organisms in the lumen. The importance of lymphoid cells in the induction of FAE was shown *in vivo* after injection of Peyer's patch (PP) lymphocytes in SCID (severe combined immunodeficiency) mice (intravenous injection, Savidge and Smith, 1995) and normal mouse (injection in intestinal mucosa, Kernéis *et al.*, 1997) resulting in the formation of new lymphoid follicles and *de novo* appearance of FAE; and *in vitro* by the acquisition of the M cell phenotype by Caco-2 cells due to the addition of PP lymphocytes (Kernéis *et al.*, 1997). The identity of the cells or factors that induce the FAE is not known but different studies suggest that B cells play an important role (Kernéis *et al.*, 1997; Golovkina *et al.*, 1999; Schulte *et al.*, 2000; Debard *et al.*, 2001).

However, whether these inductive factors act only very early in the differentiation pathway, inducing crypt cells to differentiate into FAE phenotypes or whether they can also act later to convert differentiated FAE enterocytes into M cells is still controversial. Neutra *et al.* (2001) suggest that both mechanisms of M cell formation are not mutually exclusive, but that they demonstrate the highly dynamic nature of the FAE, largely under control of the lymphoid tissue. Different authors identified cells in the follicle-associated crypts exhibiting M cell characteristics (Bye *et al.*, 1984; Gebert *et al.*, 1996, 1999; Lellouard, 2001b). Furthermore, Gebert and Posselt (1997) demonstrated that M cells in the same radial strip on the FAE display the same lectin binding patterns. This indicates

that M cells deriving from the same crypt have a common glycosylation pattern, distinct from that of M cells derived from another crypt, suggesting that a clonal population of M cells (derived from an individual crypt) migrates directly to the apex of the dome and differentiates during migration. On the other hand, evidence for conversion of FAE enterocytes into M cells is documented. Indeed, increased numbers of M cells were observed in inflamed ileal mucosa (Cuvelier *et al.*, 1994) and after bacterial exposure (Savidge *et al.*, 1991; Borghesi *et al.*, 1996, 1999). This may be attributed to the fact that bacteria provide signals to the local subepithelial immune cells in the PP, which in turn enhance the *de novo* formation of M cells. Borghesi *et al.* (1999) demonstrated that the conversion of enterocytes to M cells is restricted to the periphery of the FAE and suggest that the ability of enterocytes to undergo the conversion may depend on their stage of differentiation. Kernéis *et al.* (1997) demonstrated the acquisition of the M cell phenotype by polarised Caco-2 cells. However Lelouard *et al.* (2001b) challenged the hypothesis of conversion of FAE enterocytes into M cells. They attributed the acquisition of the M cell phenotype by Caco-2 cells to the crypt cell properties of these cells (Grasset *et al.*, 1984; 1985). They further suggested that the rapid increase in M cells after bacterial exposure may be attributed to an increase of surface area of M cells after recruitment of lymphoid cells in the pocket following antigenic stimulation; many M cells have a small surface area, which is completely covered by adjacent enterocyte microvilli, probably making them inaccessible to bacteria or microspheres and invisible by scanning electron microscopic analysis. In most murine PP and in the rabbit appendix, M cells are relatively abundant on the sides of the dome but are rare or absent in the apical region (Fujimura, 1986; Sierro *et al.*, 2000), suggesting that the M cells have here a reduced lifespan and are sloughed off earlier compared to the dome epithelial enterocytes (Gebert and Posselt, 1997). Contrary, in some mouse PP and rabbit and human PP, M cells and enterocytes are present over the entire dome and thus seem to have similar life spans.

1.3.2. M cell function

1.3.2.1. Macromolecular transport by the M cell

M cells are specialized for transepithelial transport across the epithelial barrier. Furthermore, the typical cell-surface characteristics and the local lack of surface-associated secretory IgA enhance the M cell interaction with luminal antigens. Frey *et al.* (1996) demonstrated that the enterocyte brush border glycocalyx excludes particles as small as 28 nm in diameter from contact with membrane glycolipids, whereas the M cell glycocalyx allowed close contact and endocytosis of these particles. However, 1 μm particles failed to adhere to the M cells, showing that the apical surface glycoconjugates on M cells were sufficient to prevent access of bacteria-sized particles to the membrane bilayer. However, several reports demonstrate the transcytosis of larger particles by M cells (yeast particles, 3.4 μm diameter, Beier and Gebert, 1998; particles 1-10 μm diameter, Jenkins *et al.*, 1994; Desai *et al.*, 1996).

The ability of M cells to transport macromolecules involves the directed movement of membrane vesicles. It is assumed that the membrane traffic conducted by M cells depends on the polarized organization and signaling networks typical of polarized epithelial cells (Druben and Nelson, 1996). M cells use multiple endocytic mechanisms for uptake of macromolecules, particles and micro-organisms. Adherent viruses, macromolecules and ligand-coated particles are taken up by adsorptive endocytosis via clathrin-coated pits and vesicles (Neutra *et al.*, 1987; Sicinski *et al.*, 1990; Frey *et al.*, 1996). Non-adherent materials are taken up in the fluid content of endocytic coated or uncoated vesicles (Bockman and Cooper, 1973; Owen, 1977). Large adherent particles and bacteria trigger phagocytosis. This involves the extension of cellular processes and the reorganization of the submembrane actin network similar to that seen in macrophages during phagocytosis (Neutra *et al.*, 1994a; Jones *et al.*, 1994). Each of these uptake mechanisms results in transport of foreign material into endosomal tubules and vesicles and large multivesicular bodies, located apically in the thin cytoplasmatic layer between the apical cell surface and the intraepithelial pocket (Bye *et al.*, 1984; Neutra *et al.*, 1987; Weltzin *et al.*, 1989). The large vesicles contain the late endosome/lysosome membrane

marker lgp120, generate an acidic internal milieu (Allan *et al.*, 1993) and also contain the endosomal protease cathepsin E (Finzi *et al.*, 1993), whereas the presence of other endosomal hydrolases in M cell transport vesicles has not yet been examined. Whether this intravesicular milieu alters the antigens delivered in the pocket, which may have consequences for the subsequent mucosal immune response, is not known. Furthermore, MHC II antigens on M cell membranes have been documented in subpopulations of M cells of some species (Allan *et al.*, 1993). However, it is not known whether M cells participate in the processing and presentation of antigens. Staining of the basolateral membranes, revealed that M cells have basal processes extending 10 μm or more into the underlying lymphoid tissue, where they can make direct contact with lymphoid or antigen-presenting cells. Such contacts might play a role in the induction of the unique M cell phenotype or in the processing and presentation of antigens after M cell transport (Giannasca *et al.*, 1994).

In contrast to the absorptive cells, the transeptihelial vesicular transport is the major pathway for endocytosed materials and little or no endocytosed material is directed to the lysosomes. The route of transcytotic vesicle traffic in M cells is unlike that in the absorptive cells (Gonnella and Neutra, 1984), in that only small amounts are directed to the lateral or basal cell surfaces (Giannasca *et al.*, 1994); rather all vesicles are directed to the pocket subdomain (Neutra *et al.*, 1987). The basolateral invagination brings the basolateral cell surface within a few microns of the apical membrane and greatly shortens the distance that transcytotic vesicles must travel to cross the epithelial barrier. As a consequence, the M cell mediated translocation is very efficient; the minimal transit time is only 10 min for the complete transcytosis (Owen, 1977; Neutra *et al.*, 1987). Delivery of the materials into the pocket implies that they are released from the M cell membrane during or after transport, perhaps by a change in pH or ion content in the vesicles or the pocket (Neutra and Kraehenbuhl, 1992). Introduction of latex particles (0.6-0.75 μm diameter) into ligated loops demonstrated that the particles adhered to M cells and were rapidly and synchronously transcytosed into the intraepithelial pocket so that 5% of the injected particles were taken up in a single round of endocytosis; during the following 90

min, the particles did not adhere to the M cells, suggesting that the M cell surface had been depleted of the components necessary for adherence. Whether the apical membrane components are replaced by *de novo* synthesis or by recycling from the pocket membrane is not known (Pappo and Ermak, 1989).

1.3.2.2. Antigen presentation to the lymphoid tissue following M cell transport

As the material is transported without extensive modification, the M cells are perfect antigen-sampling devices because they deliver fully antigenic luminal antigens to the immune effector cells for further processing. Following transport, the antigens are probably processed and presented by macrophages, DCs, and B cells present within or below the M cell pocket (Neutra *et al.*, 1996a). However, the actual function of cells in the M cell pocket is unknown. Yamanaka *et al.* (2001) suggest that M cell pocket memory B cells are actively engaged in sampling luminal antigens and presenting them to adjacent T cells. Activated T cells, that express CD40 ligand (CD40L), may in turn induce $CD40^+$ memory B cell survival and proliferation. Immediately below the FAE lies an extensive network of macrophages and DCs intermingled with $CD4^+$ T cells and B cells from the underlying follicle (Spalding *et al.*, 1983), which are presumably active in uptake and killing of incoming pathogens as well as processing, presentation and perhaps storage of antigens (Neutra *et al.*, 1996a). The FAE expresses a chemokine CCL20 (MIP-3α) which is not expressed elsewhere in the small intestinal epithelium (Cook *et al.*, 1994; Iwasaki and Kelsall, 2000). DCs in the subepithelial dome (SED) express the chemokine receptor CCR6 that binds to CCL20. This chemokine-receptor interaction plays a crucial role in the maintenance of this extensive DC network in the SED. CCR6-deficient mice lack this DC network in the SED and are unable to mount an immune response, although the size of their PP and the distribution of B and T cells are normal (Cook *et al.*, 2000). The DCs in this region appear to be immature. As a consequence it is likely that most antigens that are transported across the FAE by the M cells, are captured by these immature DC that subsequently migrate to the interfollicular T cell zones where they mature and present antigen (Kelsall and Strober, 1996; Iwasaki and Kelsall, 2000). During DC maturation, the CCR7 expression is enhanced which allows the DCs to migrate toward the interfolliclular

region (IFR), where high levels of MIP-3β and secondary lymphoid organ chemokine (SLC), the CCR7 chemokine ligands, are expressed (Iwasaki and Kelsall, 2000). This hypothesis is supported by different studies. In response to an injected parasite antigen, DCs migrated from the SED region to the T cell areas (Iwasaki and Kelsall, 2000). After oral administration, microparticles were captured by DCs that migrated into the underlying B-cell follicles and T cell areas in presence but not in absence of cholera toxin or attenuated *Salmonella typhimurium*, suggesting the possible need of microbial signals for this migration (Shreedhar *et al.*, 2003). *Salmonella typhimurium* was detected in DCs in the SED after oral feeding (Hopkins *et al.*, 2000); *Listeria monocytogenes* that entered the PP, became captured by DCs and later on appeared in the MLNs (Pron *et al.*, 2001), indicating that the presentation also may occur in the lymph nodes. However, functionally distinct DC subpopulations are present in the SED of which the precise role in the regulation of the nature of the mucosal immune response or oral tolerance is not clear (Huang *et al.*, 2000; Iwasaki and Kelsall, 2000; 2001).

The dose of antigen delivered by the M cell, may determine whether tolerance or a secretory immune response will be generated (Neutra *et al.*, 2001). This is consistent with following observations: 1) adherent macromolecules are effectively concentrated by adherence and may be transcytosed at least 50 times more efficiently than non-adherent material (Neutra *et al.*, 1987); 2) adherent micro-organisms, toxins and lectins readily evoke specific sIgA antibodies, whereas non-adherent commensal gut flora and soluble food antigens generally fail to do so (De Aizpurua and Russell-Jones, 1988) and may trigger a distinct differentiation and cytokine pattern in the subepithelial DCs (Iwasaki and Kelsall, 1999). Because adherent antigens elicit strong secretory immune responses, M cell adherence is thought to be a key event in induction of the mucosal immunity (Cebra and Shroff, 1994; Strober and Ehrhardt, 1994).

1.3.2.3. Selective binding and uptake of sIgA by M cells

The apical membrane of M cells selectively binds sIgA in rodents, rabbits and humans (Roy and Varvayanis, 1987; Weltzin *et al.*, 1989; Lelouard *et al.*, 1999; Mantis *et al.*, 2002) and its subsequent endocytosis and transport into the intra-epithelial pocket is

demonstrated in rodents and rabbits (Weltzin *et al.*, 1989; Mantis *et al.*, 2002). The IgA binding to M cells is not mediated by known lectin-like IgRs or by FcαRs, including CD89 (FcαRI, binding Cα2-Cα3 domains) and FcαμR (binding of IgM and IgA). Contrary, the M cell IgA receptor requires both Cα1 and Cα2 domains of IgA for binding (Mantis *et al.*, 2002). The outcome of the sIgA transport by M cells is unknown. Following transport, the sIgA-antigen complexes can be resampled in the M cell pocket or dome by APC and lymphocytes that bear Fcα receptors (Yodoi *et al.*, 1987; Mocta *et al.*, 1988). Macrophages, B cells and DC express the FcαμR that can mediate endocytosis of both IgA- and IgM-immune complexes (Shibuya *et al.*, 2000; Sakamoto *et al.*, 2001). This resampling may serve to boost the secretory immune response to pathogens that have not been effectively cleared from the lumen. Indeed, IgA-antigen complex uptake can induce secretory immune responses (Zhou *et al,.* 1995; Corthésy *et al.*, 1996). Furthermore, it has been hypothesized that by the uptake of IgA-antigen complexes, M cells sample commensal bacteria, promoting the maintenance of anti-commensal immune responses that control the luminal microflora and clear micro-organisms from the mucosa (Macpherson *et al.*, 2000; Mantis *et al.* 2002).

1.3.3. Interaction of pathogens with M cells

At mucosal sites containing M cells the risk of local invasion is high, but the occurrence of mucosal disease may be reduced by the close interactions of the FAE with antigen-processing and APCs, and by the organisation of the mucosal lymphoid tissues immediately under the epithelium. However, the M cell as antigen delivery system is exploited by some pathogens to invade the host organism. Some viruses, bacteria and protozoa exploit the facilitated transepithelial transport through M cells to invade the intestinal mucosa and cause local or systemic infections, before they can be halted by an immune response. The role of M cells in the pathogenesis of mucosal and systemic infections is extensively reviewed elsewhere (Neutra *et al.*, 1996a, 1996b; Siebers and Finlay, 1996; Sansonetti and Phalipon, 1999).

1.4. CONCLUSION

As discussed, antigens can be taken up by the intestinal epithelium by different cell types and in different ways. Insight in how antigens penetrate and translocate the epithelial layer of the gut provides clues to how antigens are presented to the local mucosal immune system. This is of particular importance in the quest to develop vaccines to prevent diseases that involves interactions at mucosal interfaces.

CHAPTER 2

THE MUCOSAL IMMUNE SYSTEM OF THE GUT: sIgA A REVIEW

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2.1. INTRODUCTION

The mucosal surface of the gastrointestinal tract (>300m^2), lined by a simple epithelium, represents a vast surface area that is vulnerable to colonization and invasion by many micro-organisms. Furthermore, it is exposed to harmless dietary components. In defense, the tissue underlying the epithelium is heavily populated with cells of the immune system, commonly referred to as the gut-associated lymphoid tissue (GALT). It is estimated that the intestinal lining contains more lymphoid cells and produces more antibodies than any other organ in the body (Mestecky and McGhee, 1987; Conley and Delacroix, 1987; Pabst and Trepel, 1975; McGhee *et al.*, 1992; Bianchi *et al.*, 1999). The GALT is anatomically and functionally divided into inductive sites, where foreign antigens are encountered and are selectively taken up for initiation of the immune response, and effector sites, more diffuse collections of lymphoid cells which comprise the effector cells for mucosal immunity (Mowat and Viney, 1997). This network is highly integrated and finely regulated and the outcome of mucosal tissue encounters with antigens and pathogens can range from mucosal and serum antibody responses and T-cell mediated immunity on one hand to oral tolerance on the other hand. This chapter highlights shortly the different cells of the inductive and the effectors sites in the GALT with a major focus on the generation and function of the secretory immunoglobulin A (sIgA).

2.2. THE INDUCTIVE AND EFFECTOR SITES OF THE GALT

The mammalian host has organised secondary lymphoid tissues in the GI tract that facilitate antigen uptake, processing and presentation for induction of mucosal immune responses, namely the Peyer's patches (PP). PP are lymphoid aggregates, consisting of multiple lymphoid follicles. The general organisation of a PP is depicted in Fig. 2.1. It is composed of a specialised follicle-associated epithelium (FAE), a subepithelial dome (SED) overlying each of multiple B-cell follicles that contain germinal centers (GCs), and interfollicular regions (IFRs), which contain high endothelial venules (HEV) and efferent lymphatics. Like other lymphoid tissues, the lymphoid cells migrating into the PP pass from the blood across HEVs present in the IFRs. The lymphoid cell components in the PP

have been analysed in several species (Ermak and Owen, 1986; Bjerke *et al.*, 1988; Ermak *et al.*, 1990).

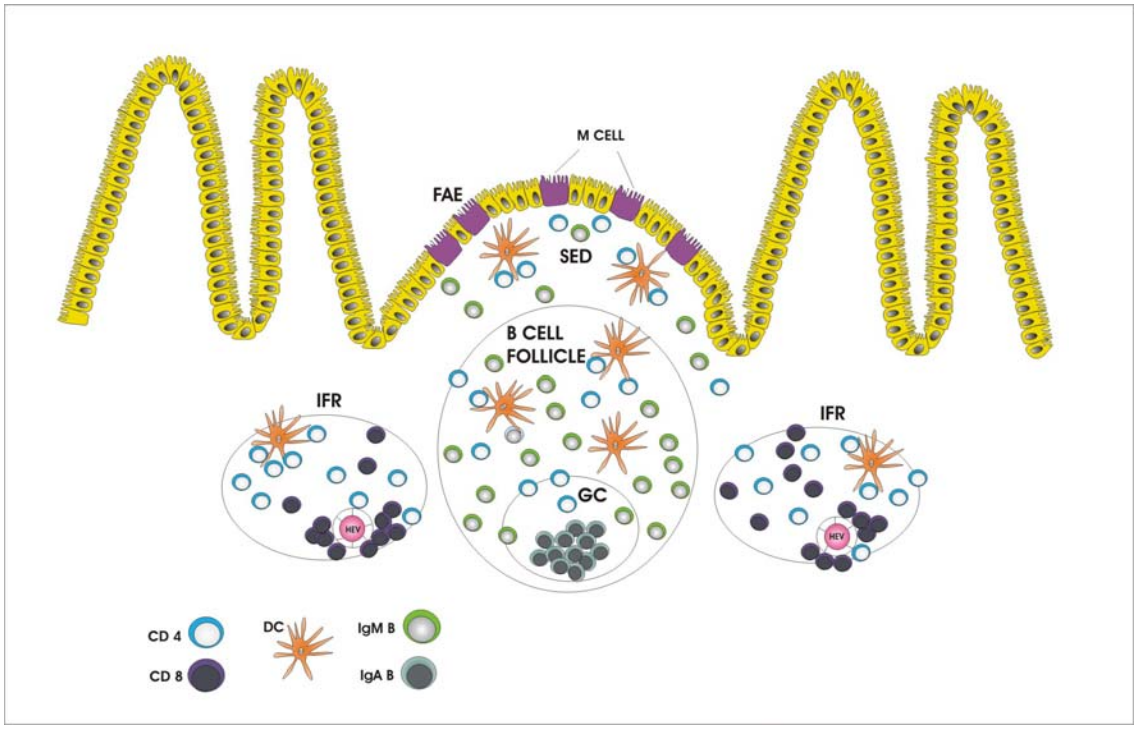

Fig. 2.1. Schematic presentation of the Peyer's patch. PP are composed of B cell follicles. Entire follicles are enriched in IgM+/IgD+ B lymphocytes, whereas germinal centers (GCs) contain mainly IgA+ lymphoblasts. Interfolliclular regions (IFRs) are enriched in T lymphocytes, mainly CD4+T cells. Dendritic cells (DCs), macrophages, and $CD4^+$T cells are scattered through the IFRs and follicles. They are particularly numerous in the subepithelial dome region (SED) situated between follicle and overlying epithelium. Follicle-associated epithelium (FAE) consists of absorptive cells and M cells specialised in antigen transport. Antigen processing and presentation is likely to occur in dome region. On antigenic stimulation, B-cells move from follicle into the GCs, proliferate and switch to expression of IgA surface receptor. Migration of cells into the mucosa takes place through the high endothelial venules (HEV), located in the IFRs.

In the FAE overlying the dome of the PP follicle, specialized antigen-sampling cells, namely the M cells are present. Luminal antigens, in the form of macromolecules, particles and micro-organisms, are transported across the epithelium into the PP via these M cells (Neutra *et al.*, 1996a).

Directly below the FAE is the SED. Studies in mouse (Kelsall and Strober, 1996), human (Spencer *et al.*, 1986), and porcine tissues (Wilders *et al.*, 1983) demonstrated that the SED is highly populated by DCs, which are likely to be the main antigen-presenting cell (APC) in this region. Macrophages are also present (Witmer and Steinman, 1984), but only in very low numbers. Furthermore, the SED contains $CD4^+$T and IgM^+B cells.

The PP B cell follicles consist of a mantle of predominantly surface IgM^+/IgD^+B cells, surrounding a basally located GC made up of B cell centrocytes and centroblasts (Weinstein and Cebra, 1991). GCs are essential for the generation of convential B2 memory cells in response to T cell dependent protein antigens, affinity maturation of the B cell receptor (somatic hypermutation) and immunoglobulin (Ig) class switching. The GC B cells are associated with a network of follicular DCs (Szabo *et al.*, 1997), scattered $CD4^+$ T cells and macrophages. Nonfollicular DCs of the immature type are scattered throughout the follicle but are rare in the GC (Kelsall and Strober, 1996). The exact function of these nonfollicular DCs is not know, but it is likely that they activate the $CD4^+$T cells and B cells (Dubois *et al.*, 1997; Fayette *et al.*, 1997), resulting in T cell help and possibly direct B cell signals necessary for B cell isotype switching to IgA (Spalding *et al.*, 1984; Spalding and Griffin, 1986). The PP B cell follicles differ from other lymphoid tissues in that GCs are always present, whereas in the other lymphoid tissues GCs are only present in times of acute infection or local immunisation. This probably reflects the continuous exposure of the PP to immunizing antigens, as germfree mice have small PP lacking GCs. In contrast to peripheral lymphoid tissue in which the main Ig-isotype produced is IgG, the PP GCs contain high numbers of surface-IgA-expressing B cells (McGhee *et al.*, 1989). A preferential switch to IgA occurs, due to the microenvironmental conditions existing in the PP (Murray *et al.*, 1987).

The IFRs are marked by a network of mature DCs and macrophages (Kelsall and Strober, 1996) and the presence of T cells and by the paucity of B cells. Most of the T cells in this region (70%) are $CD4^+$T cells. $CD8^+$T cells are also present in the IFR, but are restricted to a narrow band in the central portion of this region.

Following uptake by the M cells and transport to the SED, the antigen encounters DCs, macrophages, $CD4^+$T cells and B cells. Here, initial cognate interactions occur between APC and T cells, or T cells and B cells (Ermark and Owen, 1986). Furthermore, immature DCs at this site phagocytose invading micro-organisms or take up soluble antigens, after which they migrate to IFRs or into the B cell follicles to initiate immune responses. All together, these interactions may lead to the generation of Th1 (T-helper 1)

cells (involved in the activation of macrophages and essential component of the defense against intracellular organisms), Th2 cells (essential for the generation of plasmacells), TGF-β producing cells, including the Th3 cells and $CD8^+$ suppressor cells, IL-10 producing T regulatory type 1 (Tr1) cells (involved in the generation of oral tolerance), cytotoxic T lymphocytes (CTL, essential for defence against intracellular organisms) and antibody-producing plasmacells (essential for defence against extracellular pathogens) (McGhee *et al.*, 1992).

Following induction in the PP, the antigen-activated and memory B and T cells emigrate form the inductive environment via lymphatic drainage, circulate through the bloodstream to reach the spleen, and home to mucosal effector sites.

The main effector sites of the intestinal immune responses are the intestinal lamina propria (LP), and the epithelium (McGhee *et al.*, 1992). However, the effector cells can also home to other mucosal and glandular sites, including those of the oral cavity (salivary glands), lacrimal glands, the respiratory and genitourinary tract, and lactating mammary glands. This pathway links the several mucosa and has led to the concept of a common mucosal immune system (Bienenstock *et al.*, 1978; Mestecky, 1987; Brandtzaeg *et al.*, 1998).

Once in the LP, the T cells are present in a dormant state as resting memory cells and on re-encounter with antigen they express their definitive effector functions (Khoo *et al.*, 1997; Bailey *et al.*, 1998; Haverson *et al.*, 1999), such as the production of helper or suppressor cytokines, or the mediation of cytotoxicity, whereas the effector B cells differentiate into mainly IgA-producing plasmacells (Neutra *et al.*, 1994b). In the pig, plasma cells and B cells predominate around the crypts, and T cells in the villi. The $CD8^+$T cells are found immediately below the epithelial cells and adjacent to the basement membrane, whereas the $CD4^+$T cells are present in the core of the villi (Vega-Lopez *et al.*, 1993). Beside these effector cells originating in the PP, the LP contains high numbers of macrophages and DCs (professional APC), whereas neutrophils, eosinophils, basophils and mast cells are also regularly found, albeit in low numbers (Pabst and Beil, 1989; Stokes *et al.*, 1994, 1996; Haverson *et al.*, 1994; 1999; 2000).

In addition to the LP, the effector lymphocytes can home to the epithelium, known as the intraepithelial lymphocytes (IELs). In pigs, the number of IELs increases from 2.6% of all epithelial cells on day 1 to 30% at 2 months (Pabst and Rothkötter, 1999). Most IELs are T cells (Bjerke *et al.*, 1988) which comprise over 90% in pigs; 77% are $CD8^+$ and 5% $CD4^+$ (Stokes *et al.*, 1994). $CD4^-/CD8^-$T cells comprise a small proportion of the IELs in adult pigs (3 to 4% at the age of 14 months), whereas this proportion is high in very young pigs (± 30% at the age of 5 days) (Rothkötter *et al.*, 1994).

In mice, two populations of $CD8^+$ intraepithelial T cells exist. One $CD8^+$T cell population express the heterodimeric αβCD8 chains and TCRαβ, and represent the progeny of T lymphoblasts elicited in the PP by antigen stimulation. The other $CD8^+$T cells bear homodimeric ααCD8 chains and TCRαβ or -γδ. These ααααCD8$^+$T cells recognise an antigenic repertoire different from the αβCD8$^+$T cells (Guy-Grand *et al.*, 1991a). The TCRγδ- bearing ααCD8$^+$T cells may recognise antigens (proteins and non-protein determinants, like phosphoproteins and carbohydrates) presented by nonclassical MHC class I molecules, like CD1. The TCRαβ- bearing ααCD8$^+$T cells may recognise some superantigens presented in a nonclassical way on the MHC class II molecules or antigens bound to CD1 molecules (Guy-Grand *et al.*, 1991a). The ααCD8$^+$T cells may be of thymic origin (Guy-Grand *et al.*, 2001, 2003) or may be produced in the gut by cryptopatches (Ishikawa *et al.*, 1999; Makita *et al.*, 2003). Whereas αβCD8$^+$T cells are also present in the LP, this is not the case for ααCD8$^+$T. The actual functions of these IELs *in vivo* are not yet known; they are cytotoxic after activation and probably ensure the epithelial integrity by rapid killing injured or virus-infected cells that are eliminated from the epithelium afterwards (Guy-Grand *et al.*, 1991b). In contrast to mice, in pigs, no MAC-320$^+$ lymphocytes which are preferentially γδT cells were detected in the epithelium (Rothkötter *et al.*, 1999).

In addition to the PP, other organized lymphoid tissues have been described. Isolated lymphoid follicles in the mucosa and submucosal lymphocyte aggregations have been described in human, mouse, rabbit and guinea pig and are secondary lymphoid tissues representing solitary PP follicles (Moghaddami *et al.*, 1998; Hamada *et al.*, 2002).

Kanamori *et al.* (1996) described aggregations of 1000 lymphocytes in the LP crypt of the small and the large intestine of mice, the cryptopatches. These sites act as primary lymphoid organs where intraepithelial T lymphocytes expressing the CD8αα homodimer develop *de novo* from bone-marrow-derived precursors (Saito *et al.*, 1998; Oida *et al.*, 2000). In mouse, rat and human, specialized intestinal villi, called the lymphocyte-filled villi, have been described (Moghaddami *et al.*, 1998). In rodents, they are hypothesized to be extra-thymic sites of primary T-cell differentiation, where luminal antigens may play a role in repertoire expansion and/or selection. In humans, they are not the functional homologues of those in mice and rats, but are probably secondary lymphoid tissues, involved in presenting antigens to memory T cells. Organized lymphoid structures other than PP have not been described in the pig until now (Pabst and Rothkötter, 1999).

It is important to point out here that the distinctions between inductive and effector sites, while generally applicable, are not absolute. Certain responses can be induced in the gut epithelium and LP, and some effector cells might operate in the PP (Kelsall and Strober, 1999). In the effector tissues, antigen uptake and presentation can occur. Intact antigens can transverse the epithelial barrier, by the paracellular or intracellular pathway, after which intact antigen may be processed for induction of B- and T-cell responses. MHC class II^+ surface IgA^+B cells may process and present peptides to $CD4^+$Th cells; also macrophages and DCs (Mayrhofer *et al.*, 1983; Pavli *et al.*, 1990; 1993; Liu and Macpherson, 1995; Haverson et al., 2000) in the LP may present such antigens. Yamamoto *et al.* (2000) demonstrated that oral immunisation of mice lacking PP resulted in antigen-specific mucosal IgA and serum IgG responses which were induced in the MLNs and the spleen. In contrast, neither mucosal nor serum antibodies (Abs) were induced after oral immunisation of mice lacking both PP as well as MLN, demonstrating the importance of the MLNs for induction of both mucosal as serum antibody (Ab) responses after oral immunisation.

In the pig, Peyer's patches are present in both the jejunum as ileum (Binns and Licence, 1985). The jejunal PP (JPP) are distributed as discrete patches along the jejunum and proximal ileum and persist throughout life. On the contrary, the ileal PP (IPP) occurs

as a single continuous patch, commencing near the ileo-caecal junction and extending for up to 2 m along the terminal ileum but shrinks within a year to form discrete patches. These two types of PP differ in structure, development, lymphocyte migration and production (Pabst *et al.*, 1988; Barman *et al.*, 1997). Whereas, the porcine JPP is a secondary lymphoid tissue, the pig IPP is like the sheep IPP, considered as a primary lymphoid organ generating the primary B lymphocyte repertoire and producing the systemic B lymphocyte pool (Andersen *et al.*, 1999). However, Pabst and Rothkötter (1999) suggest that the last part of the ileal patch before the ileocaecal junction, resembles the jejunal patches, based on difference in subset composition, lymphocyte traffic and morphology along the IPP (Binns and Pabst, 1988; Zuckermann and Gaskins, 1996).

2.3. SECRETORY IGA

IgA represents the most prominent antibody in the LP and is the best defined effector component of the GALT (Brandtzaeg *et al.*, 1999). In humans, 80-90% of the terminally differentiated B cells found in the LP are IgA immunocytes (blasts and plasma cells) that produce polymeric IgA, mainly as dimers (Brandtzaeg, 1974). In man approximately 3 g of IgA is delivered each day into the intestinal lumen (Conley and Delacroix, 1987). The predominance of IgA antibodies in mucosal sites reflects a combination of high rate IgA isotype switching among precursor cells in inductive sites (Beagley *et al.*, 1988; 1989), their selective homing to mucosal effector tissues (Abitorabi *et al.*, 1996) and vigorous proliferation of these cells after extravastion (Husband and Gowans, 1978) and differentiation towards IgA-producing plasma cells.

2.3.1. Generation of IgA-producing plasma cells

B cell activation by protein antigen requires binding of the antigen to the B cell surface immunoglobulin - the antigen-specific B cell receptor (BCR) - and also requires costimulation by antigen-specific T cells through CD40-CD40 ligand interaction and the secretion of cytokines. Appropriately activated B cells proliferate and differentiate to plasma cells or to long-lived memory cells, and it is during this differentiation process that B cells use unique strategies for further diversifying the BCR repertoire. This is achieved by somatic hypermutation and class-switch recombination. The BCR is retained

when the cell enters the memory pathway, but it is gradually lost (together with several other B cell markers, such as murine B220) during plasma cell differentiation (Brandtzaeg *et al.*, 2001).

The PP are specialized for the induction of antigen-specific IgA lymphoblasts (Craig and Cebra, 1971; McGhee *et al.*, 1992). Within the PP, the specialized GC microenvironment, which allows strong interactions between B cells, antigens trapped on follicular DCs and local $CD4^+$T cells, facilitates B cell proliferation, class-switch recombination to IgA^+B cells and somatic hypermutation (Weinstein and Cebra, 1991). Although the PP are specialized for the induction of IgA^+B cells, terminal differentiation and secretion is not taken place there. In the PP, IgA-secreting plasma cells are relatively absent (Brandtzaeg and Baklien, 1976) as is the receptor-mediated system for IgA export in the FAE (Pappo and Owen, 1988). Following induction in the PP, the IgA^+B cells pass through the MLNs, where they proliferate further and differentiate into plasmablasts. Subsequently, they circulate via the lymphatic system to enter the subclavian vein from the thoracic duct, after which they home back via the arterial blood to the intestinal LP (Husband and Gowans, 1978). However, there is a major difference in lymphocyte circulation in the pig compared with other species (Binns, 1982). Whereas in other species, the lymphocytes exit the lymph nodes via the efferent lymphatics, the porcine efferent lymph contains very few lymphocytes. Instead, the lymphocytes in the lymph nodes directly re-enter the circulation via the HEV (Pabst and Binns, 1989). The tissue specificity of IgA^+B cell homing is the result of complex interactions between receptors that are present on the lymphocytes and their ligands expressed on the vascular endothelium of the target tissues. The $\alpha 4\beta 7$ integrin expressed by lymphocytes and the mucosal vascular addressin cell adhesion molecule 1 (MADCAM1) expressed by blood vessels in the LP form the main receptor-ligand pair that is required for the homing of lymphocytes to the LP (Briskin *et al.*, 1993; Berlin *et al.*, 1993). Although this interaction is important for mucosal lymphocyte homing, it can not explain the preferential homing of circulating precursors of IgA^+, but not IgM^+ or IgG^+ plasma cells to the gut LP. In mice, CCL25, also known as the thymus expressed chemokine (TECK), is probably one of the

chemokines responsible for the selective migration of circulating IgA^+B cells to the intestinal LP (Bowman *et al.*, 2002). CCL25 is mainly produced by the epithelium of the small intestine (Kunkel *et al.*, 2000; Papadakis *et al.*, 2000) and IgA^+, but not IgM^+ or IgG^+plasma cells migrate in response to CCL25, owing to the selective expression of the CCL25 receptor (CCR9) on IgA^+ plasma cell precursors (Bowman et al., 2002). Furthermore, signalling through the lymphotoxin β receptor (LTβR) on LP stromal cells is absolutely necessary for the presence of IgM^+B cells and IgA^+ plasma cells in the LP (Kang *et al.*, 2002; Newberry *et al.*, 2002). Impaired LTβR signalling might result in a decrease in the local concentration of adhesion molecules and chemokines, causing the absence of LP B cells (Fagarasan and Honjo, 2003). However, the molecular mechanisms by which lymphotoxin (LT)-LTβR interactions selectively affect B cell homing to the gut LP are not resolved.

Terminal differentiation occurs in the effector site, the LP, that is the major site of IgA production. The terminal differentiation is driven by factors in the mucosal environment, including cytokines and especially IL-6 and IL-5 (Matsumoto *et al.*, 1989; Beagley *et al.*, 1991; Husband *et al.*, 1996). It has been shown *in vitro* that TGF-β and IL-4 promote the switch form IgM to IgA surface expression and subsequent IgA secretion (Kunimoto *et al.*, 1988; Coffman *et al.*, 1989). Once the switch has taken place, IL-5 (Kunimoto *et al.*, 1988; Beagley *et al.*, 1988) and IL-6 (Beagley *et al.*, 1988; Kunimoto *et al.*, 1989) act to enhance secretion of IgA (McGhee *et al.*, 1992). IL-10 synergises with TGF-β to increase the efficiency of IgA switching (Defrance *et al.*, 1992). The importance of these cytokines has been confirmed by different *in vivo* studies (Husband *et al.*, 1996; 1999).

2.3.2. Structure and secretion of sIgA

All immunoglobulin isotypes consists of two heavy (H) and two light (L) chains, but for IgA, this H_2L_2 monomeric unit can polymerise further. Mucosally produced IgA consists predominantly of dimers and some larger polymers (trimers and tetramers), collectively called polymeric IgA (pIgA) (Brandtzaeg *et al.*, 1999). IgA polymerisation is regulated by the incorporation of a 15 kDa polypeptide, the joining chain (J chain) in that

its presence greatly stimulates polymerisation (Johansen *et al.*, 2001) and is directed by the COOH-terminal domains of the heavy chains (Braathen *et al.*, 2002). The J chain is synthesized along with IgA in plasma cells. J chain incorporation is an early event in IgA polymerisation and this peptide is found in all polymeric forms of this isotype (Vaerman *et al.*, 1995; Sørensen *et al.*, 2000). Polymerisation of two or more IgA molecules with the J chain occurs late in the secretory pathway, just before release from plasma cells (McCune *et al.*, 1981). It is often assumed that pIgA contains only one J chain molecule (Zikan *et al.*, 1986), but immunochemical studies indicate that dimeric IgA contains two J chains (Brandtzaeg, 1975; Grubb, 1978) and Vaerman *et al.* (1995) suggest that the molar J chain ratio increases with the size of the polymer.

The central role of pIgA in protecting the mucosal surface relies on the existence of an active mechanism used to translocate pIgA through the intestinal enterocytes and to transfer it to the intestinal lumen. The translocation mechanism depends on a 110-kDa transmembrane glycoprotein expressed by the enterocyte, termed the polymeric immunoglobulin receptor (pIgR) (Mostov, 1994). The extracellular part of this pIgR contains 5 Ig-like domains (D1 to D5). In humans, the binding of pIgA is initiated by non-covalent interaction at D1, further non-covalent interactions with D2 and/ or D3 allow stable binding, after which this complex is stabilized by covalent interactions with D5 by means of disulfide bonds (Fallgreen-Gebauer *et al.*, 1993; Norderhaug *et al.*, 1999b). Both the J chain (Vaerman *et al.*, 1998; Johansen *et al.*, 2001) as the COOH-terminal domain of the heavy chains (Braathen *et al.*, 2002) are essential for binding pIgA on the pIgR. The pIgR is internalized by endocytosis from the basolateral membrane into basolateral endosomes and subsequently sorted for transcytosis across the enterocytes (Apodaca *et al.*, 1991; Schaerer *et al.*, 1991). This transport occurs continuously independent of binding of pIgA. At the apical cell surface, the receptor is cleaved by a cell-surface associated serine protease at the junction between the extracellular domain and the membrane-spanning region and the extracellular part of the receptor, the secretory component (SC) free or attached to pIgA, is released into the intestinal lumen (Brandtzaeg *et al.*, 1999; Norderhaug *et al.*, 1999a). The signals controlling basolateral

targeting, endocytosis and sorting of the receptor for transcytosis in basolateral endosomes involve tyrosine-containing tight beta turns and phosphorylation sites in the receptor's cytoplasmic tail (Apodaca *et al.*, 1991; Schaerer *et al.*, 1991; Hirt *et al.*, 1993). Sequence comparison of the pIgR gene of rabbit (Mostov *et al.*, 1984; Schaerer *et al.*, 1990), rat (Banting *et al.*, 1989) and human (Krajei *et al.*, 1989) indicate that the intracellular targeting signals on the tail are highly conserved. Expression of the pIgR gene on epithelial cells is upregulated *in vitro* by several cytokines, such as IFN-γ (Sollid *et al.*, 1987). The covalent attachment to the SC stabilizes sIgA in the secretions by making it more resistant to proteases (Lindh, 1975; Crottet and Corthesy, 1998).

In addition to the transepithelial transport of pIgA by the enterocytes, pIgA can also be delivered into the intestinal lumen through secretion into bile, which is released into the duodenum via the ductus choleduchus through the ampulla of Vater at the major duodenal papilla. To be delivered into the bile, pIgA is either transported across hepatocytes (Orlans *et al.*, 1978; Jackson *et al.*, 1978), which express the pIgR, into biliary canaliculi such as is the case in rodents and chickens (Orlans *et al.*, 1979; Kuhn and Kraehenbuhl, 1981), or across the biliary epithelia of the bile ducts and gall bladder (Nagura *et al.*, 1983; Vuitton *et al.*, 1985; Tomana *et al.*, 1988) in species whose hepatocytes lack pIgR, such as humans (Delacroix *et al.*, 1982; Dooley *et al.*, 1982) and pigs.

2.3.3. Mechanisms of sIgA-mediated protection

IgA can perform antibody-dependent-cell-mediated-cytotoxicity (ADCC) and promote phagocytosis via FcαRI (CD89) receptors on cells of the myeloid lineage including monocytes, macrophages, neutrophils and eosinophils (Tagliabue *et al.*, 1984). Furthermore, FcαRI facilitates antigen presentation on human DCs (Geissmann *et al.*, 2001). IgA can further induce respiratory burst activity by polymorphonuclear leukocytes and trigger eosinophil and basophil degranulation (van Egmond *et al.*, 2001). However, the major biologic activities of IgA would appear to be non-inflammatory. This is accentuated by the fact that IgA is a poor activator of complement. Although IgA can trigger, under select conditions, the alternative complement pathway (Janoff *et al.*, 1999),

it can not bind C1q, therefore it can not activate the classical pathway (Kerr, 1990). This may be of importance in the maintenance of integrity of mucosal surfaces. Indeed, activation of complement induces local inflammatory reactions, including the influx of polymorphonuclear leukocytes and release of substances, like cytokines, that enhance the permeability of mucosal membranes (McGhee *et al.*, 1992).

IgA antibodies secreted by plasma cells in the LP can potentially bind antigens in different locations relative to the mucosal epithelium (Lamm, 1997). They can complex with antigens present locally in the LP. These immune complexes can either be taken up by phagocytic cells or be absorbed into the vascular system, or be transported across the epithelium into the lumen by the same pIgR-mediated path utilized by free pIgA (Kaetzel *et al.*, 1991). By doing so antigens that leak through the epithelial barrier can be cleared back into the lumen, and monomeric IgA or IgG antibodies, which themselves are not ligands for the pIgR, can be transported as part of these immune complexes (Kaetzel *et al.*, 1994). This immune elimination role of IgA might provide an effective means of ridding the mucosal tissues of (excessive) immune complexes. Furthermore, during the pIgR-mediated transport process specific IgA can bind to newly synthesized viral proteins inside the epithelial cells, preventing virion assembly and neutralizing viral replication (Mazanec *et al.*, 1992, 1995; Bomsel *et al.*, 1998; Fujioka *et al.*, 1998). Prevention of virion assembly and budding by IgA acting intracellularly may potentially forestall cytopathic effects, so spare the cell and be a mechanism for recovery from infection. This preservation of the integrity of the mucous membrane could help to maintain the epithelial barrier and to retard systemic dissemination of viral antigens. Finally, IgA can interact with antigens within the lumen after epithelial transcytosis. IgA antibodies can thereby interfere with the ability of antigens, including viruses as well as bacteria and their toxins and enzymes, to adhere to and penetrate the mucosa, a phenomenon called 'immune exclusion' (Neutra *et al.*, 1994b; Brandtzaeg, 2003). Because of the polymeric nature of sIgA, it displays greater avidity than monomeric Ig and can efficiently crosslink target macromolecules and micro-organisms, thereby inhibiting motility and facilitating entrapment in mucus and clearance by peristalsis (McGhee *et al.,* 1992; Renegar *et al.*,

1998). Furthermore sIgA is a hydrophilic, negatively charged molecule because of the predominance of hydrophilic amino acids in the Fc region of IgA, and abundant glycosylation of both IgA and SC (Kerr, 1990). Consequently, micro-organisms surrounded by sIgA will be repelled by the mucosal surfaces. Terminal mannose-containing oligosaccharide side chains on especially human IgA_2 heavy chains are recognised by mannose-specific lectins present on type 1 fimbriae. Thus, these carbohydrate-specific interactions represent an important protective anti-adherence function of sIgA against bacteria, regardless of the specificity of the IgA molecule (McGhee *et al.*, 1992). In other cases, sIgA can directly block the microbial sites that mediate epithelial attachment, either by binding to specific adhesins or by sterically hindering their interaction with epithelial cells (Williams and Gibbons, 1972; Svanborg-Eden and Svennerholm, 1978).

2.3.4. Distribution of the sIgA response

Uptake and sampling of antigens in the mucosal immune system occurs locally, at the specific inductive sites. Contrary, the secretory immune response to antigens may be detected both at the site of initial sampling and in distant mucosal and glandular tissues. This is due to the selective migration of effector and memory cells into subepithelia and glandular connective tissues throughout the body where they differentiate into plasma cells that produce IgA. The existence of this common mucosal immune system is well documented in both experimental animals (Weisz-Carrington *et al.*, 1979; McDermott and Bienenstock, 1979; Mestecky, 1987; Saif, 1996) and humans (Czerkinsky *et al.*, 1987; Quiding *et al.*, 1991). However, the IgA response is highly regionalized in that the local response of mucosal immunization is consistently greater than the diffuse response at distant sites (Husband and Gowans, 1978; Husband, 1982; Pierce and Cray, 1982; Haneberg *et al.*, 1994) either as a result of some direct migration or of selective homing after circulation via the blood to the area of the intestine where antigen was encountered. After intra-intestinal boosting of intraperitoneal (i.p.) primed rats, the greatest density of antibody containing cells consistently occurred at, or distal to, the boosted site. This was not due to antigen-induced activation or multiplication of precursors within the LP but to

migration of plasma cells to these sites (Pierce and Gowans, 1975). Husband and Dunkley (1985) demonstrated that the homing of plasma cells to the area of antigen encounter is not due to preferential homing to their site of origin (i.e. precursors arising from challenge in the jejunum, do not preferentially home to the jejunum, but home to the same extent to the different parts of the intestine). While the site of origin may affect extravasation, the overriding influence on ultimate distribution patterns is the site of antigen challenge inducing local retention and proliferation. Administration of cholera toxin (CT) into the proximal intestine, distal intestine or colon of rat evoked highest anti-CT IgA in the segment of antigen exposure (Pierce and Cray, 1982). Similarly, restriction of the antigen administration to either the stomach or the trachea in mice, resulted in concentrated secretory immune responses in either the digestive tract or the airways, but not in both (Nedrud *et al.*, 1987). This regionalisation of the immune response may be functionally important as it concentrates the production of sIgA at the site of potential microbial colonisation or invasion. The mechanism(s) whereby the sIgA response is concentrated in the region of luminal antigen load is not known. Kraehenbuhl and Neutra (1992) hypothesised that initial uptake of antigen at any inductive site stimulates production and dissemination of antigen-specific IgA memory cells into mucosae and glands throughout the body via the common mucosal immune system but that subsequent local entry of the same antigen resulting in local cytokine release affects local IgA memory cells and results in a local secondary response seen as production and differentiation of IgA–producing plasma cells.

2.3.5. B1 lymphocytes

Recent studies in mice challenge the view that Peyer's patches are the main inductive site for the generation of IgA^+ plasma cells, since cellular interactions outside Peyer's patches in the gut LP are important for the induction of IgA responses (Fagarasan and Honjo, 2003). Indeed, B cells are not only produced in the bone marrow (B2 cells), but also in the peritoneal cavity (B1 cells) (Solvason *et al.*, 1991). In mice, it has been shown that much of the serum IgM and 40-50% of the intestinal IgA is derived from B1 cells (Kroese *et al.*, 1989). B1 cells appear to produce antibodies in a T cell-independent

manner. These cells, originally defined by the surface expression of CD5 and high levels of IgM, have a capacity of self-renewal. B1 cells play an important role in innate immunity by secreting large amounts of "natural" antibodies of the IgM class. These IgMs have been considered as "natural" antibodies, since they circulate in the blood of non-immunized mice and are produced in germ-free conditions when the cognate antigens are presumed to be absent; therefore, they are produced without exposure to any environmental antigens or immunization. These antibodies are relatively undiversified as they lack the molecular hallmarks of having been through a GC reaction, namely the mutations in the complementarity-determining regions of the antibody variable domain (Förster *et al.*, 1988; Tarlinton *et al.*, 1988). The resultant antibodies are highly cross-reactive and bind with low affinity to self-antigens and common bacterial antigens (polysaccharides and polymers with repetitive subunits, such as bacterial wall components) (Hayakawa *et al.*, 1984, 1986).

In contrast to the studies in mice, conclusive evidence for a supply of B1 cells form the peritoneal cavity (PC) to the intestinal LP does not exist in other species (Brandtzaeg, 2001), although analogous cells have been reported in human (Solvason and Kearney, 1992), sheep (Gupta *et al.*, 1998a) and pigs (Cukrowska *et al.*, 1996; Appleyard and Wilkie, 1998). In mice, 40 to 50 % of the LP IgA^+B cells are derived from the PC (Kroese *et al.*, 1989). These IgA^+B cells are generated from IgM^+B1 cells without GC involvement. Instead, isotype class switching and differentiation to IgA^+plasma cells are occurring *in situ* in a T-independent manner. This IgA switching and differentiation into plasma cells are facilitated by a unique microenvironment created by cytokines derived from LP stromal cells. Since in the LP, antigen presentation might occur by DCs (Rescigno *et al.*, 2001b), the interaction between LP B1 cells, DCs and stromal cells would explain the T cell-independent induction of IgA synthesis (Fagarasan *et al.*, 2001). Although B1 cells are generally encoded by unmutated germline immunoglobulin variable (IgV) genes, murine B1 cells producing IgA in response to commensal bacteria sometimes show somatic hypermutation, indicative of an antigen-driven selection process (Bos *et al.*, 1996). This selection of IgA^+B cells would also take place *in situ* by antigens captured by DCs

(Fagarasan and Honjo, 2003). On the other hand, it is possible that the proliferation and differentiation to IgA^+plasma cells take place in the MLN (Fagarasan and Honjo, 2000).

The T cell-independently induced IgA antibodies against cell wall antigens and proteins of commensal bacteria (derived mostly from B1 cells), are not simply a "natural" antibodies since they are specifically induced in response to their presence within the commensal gut flora, as shown after recolonising T cell deficient mice with bacteria producing a novel protein (Macpherson *et al.*, 2000). Consequently, the induction of anti-commensal sIgA is antigen driven. These B1 cell-derived IgA antibodies play an important role in host defences at the mucosal surface, preventing systemic invasion by commensal bacteria. Indeed, commensal bacteria are bound by B1 cell-derived and to a lesser extend by B2 cell-derived intestinal IgA (Bos *et al.*, 1996) and normal mice have intestinal B1 cell-derived IgA specific for commensals but lack commensal-specific IgA or IgG antibodies in the serum (Macpherson *et al.*, 2000). By contrast, mice with IgA deficiency do have serum IgG that is specific for commensal bacteria, but this IgG is produced by B2 cells in a T cell-dependent manner (Macpherson *et al.*, 2000). The commensal bacteria that have crossed the mucosal barrier would be coated by commensal-specific IgA and either be transported back into the lumen by pIgR-mediated transcytosis (Kaetzel *et al.*, 1991) or taken up locally and degraded by macrophages expressing μ/α Fc receptors (Shibuya *et al.*, 2000; Sakamoto *et al.*, 2001).

2.3.6. Other antibodies present in the LP.

IgA is overwhelmingly the most important immunoglobulin in the intestine; however, the other isotypes (IgM, IgG or IgE) are also locally produced in relatively small amounts (<10%), whereas their proportion may increase in inflamed or diseased tissues (Macpherson *et al.*, 1996) or in cases of IgA deficiency in which case mucosal IgM production and secretion partly compensate for the lack of IgA (Friman *et al.*, 1994; Harriman *et al.*, 1999). However, in the pig IgM is more a mucosal isotype compared with other species and both IgM as IgA are important isotypes in the mucosal lymphoid organs especially in the young pig. Bianchi *et al.* (1999) have studied the postnatal development of the isotype specific Ig-secreting cell response in various lymphoid organs of specific

pathogen free (SPF) pigs between 1 and 40 weeks of age. In these SPF-pigs, the shift from IgM to IgA as predominant mucosal isotype was first observed in the duodenum and jejunum (12 weeks) and later in the ileum (40 weeks). As antigen exposure may influence the development of the Ig-secreting cell repertoire, the frequencies of Ig-secreting cells in the mucosal lymphoid organs were compared between SPF-pigs and conventional pigs at the age of 40 weeks. At that age, no differences were found between SPF-pigs and convential pigs concerning the amount of IgM and IgA in the intestine (Bianchi *et al.*, 1999). Like IgA, IgM has the ability to polymerise, mainly to pentamers with incorporated J chain and to hexamers without (Brewer and Corley, 1997; Sørensen *et al.*, 2000). The latter activate the complement cascade up to 20-fold more efficiently than pentameric IgM (Randall *et al.*, 1990; Wiersma *et al.*, 1998). Whereas monomers are also secreted form IgA-producing plasma cells, monomers are mainly retained and degraded in IgM-producing plasma cells (Sitia *et al.*, 1990). The J chain can be produced by immunocytes of all Ig isotypes, but it becomes incorporated only into pIgA and pentameric IgM; in their absence, the J chain is subjected to intracellular degradation (Brandtzaeg, 1985). In contrast to IgA, the J chain is incorporated late in the polymerisation process, making polymerisation possible without incorporation of the J chain (Brewer and Corley, 1997). As for IgA, only J chain containing IgM polymers bind the pIgR and are transported across the epithelium (Brandtzaeg and Prydz, 1984). However, the mode of pIgR binding differs between pIgM and pIgA and in some species, most notably the rabbit, the ability of the pIgR to bind pentameric IgM has selectively been lost (Røe at al., 1999). Contrary, pIgM and pIgA are bound by the human pIgR with similar affinity (Natvig *et al.*, 1997; Røe *at al.*, 1999). However, the external transport of pIgA is normally favoured over pentameric IgM because of its dominant local production and its better access to the pIgR by enhanced diffusion through the extracellular matrix and epithelial basement membranes (Natvig *et al.*, 1997).

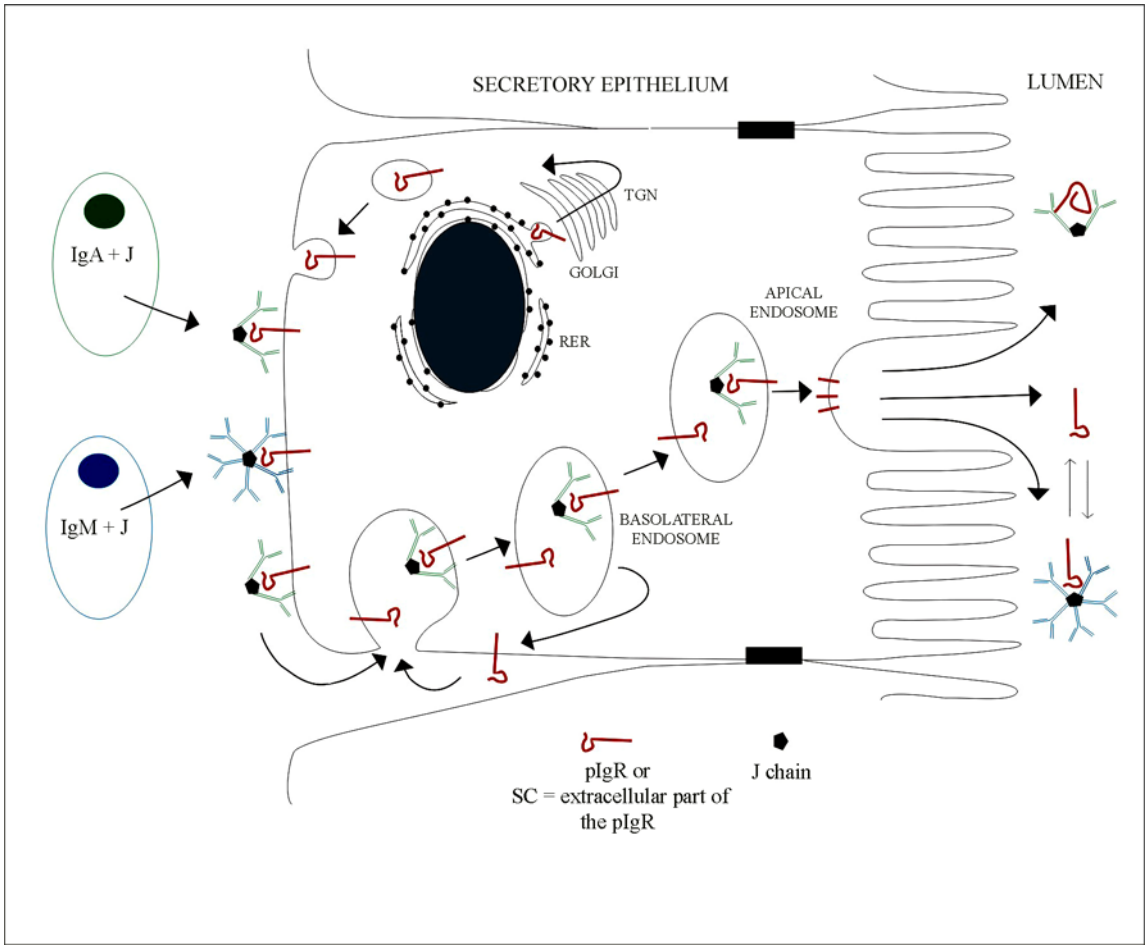

Fig. 2.3. Schematic representation of the various steps involved in the generation of sIgA and sIgM via pIgR-mediated epithelial translocation. The transmembrane secretory component (SC) or pIgR is synthesised in the rough endoplasmatic reticulum (RER) of the secretory epithelial cells and matures by terminal glycosylation in the Golgi complex. After being sorted through the trans-Golgi network (TGN), it is phosphorylated and expressed as pIgR at the basolateral plasma membrane. Endocytosis of ligand-complexed and unoccupied pIgR is followed by transcytosis to apical endosomes and finally by cleavage and release of the secretory Ig molecules with bound SC, as well as excess of free SC, at the luminal cell face. Some basolateral recycling may initially take place for unoccupied pIgR as indicated. During the external translocation, covalent stabilisation of the IgA/SC complexes regularly occurs (disulfide bridge indicated in sIgA), whereas the free SC in the secretion apparently serves to stabilise the non-covalent IgM/SC complexes (dynamic equilibrium indicated for sIgM). Adapted form Brandtzaeg *et al.* 1999.

IgG can not be transported by the pIgR. However, it may provide mucosal protection by paracellular passive transfer (Norderhaug *et al.*, 1999a). Furthermore, a bidirectional transport mechanism for IgG via the MHC class I-related Fc receptor, FcRn is demonstrated in a human intestinal epithelial cell line (Dickinson *et al.*, 1999), raising the possibility that IgG may also be actively transferred to secretions.

Whereas the capillaries in the PP mucosa are non-fenestrated and relatively impermeable to serum antibodies (Allan and Trier, 1991), making the PP and FAE

functionally isolated from blood-borne factors, the villous capillaries are highly fenestrated allowing diffusion of serum proteins into the LP. As a consequence, serum-derived antibodies can be found in the LP. Like IgA locally produced in the LP, serum-derived IgA can be selectively transported into the intestinal lumen via the pIgR. Sheldrake *et al.* (1984) hypothesized an inverse relationship between the level of local production of IgA by plasma cells underlying secretory epithelia and the amount of serum-derived IgA in secretions. Consequently, at sites with lesser numbers of IgA plasmacells and therefore reduced opportunity for local IgA antibody production, there is a concomitant increase in selective transport of IgA from serum into mucosal secretions, providing a compensatory mechanism to ensure that IgA antibody reaches mucosal secretions even in the absence of extensive local production. This selective transport of serum IgA at the mucosal sites is probably dependent on secretory component availability. Where local IgA production is predominant, there are fewer SC molecules available for serum transport into the mucosal secretions. In the pig, 22.7-29.5% of IgA in the gut lymph comes from serum, versus 77.3 to 70.5% from local production in the intestine (Vaerman *et al.*, 1997). Conversly, 31% of the total plasma IgA originates from local intestinal synthesis, reaching the blood via mesenteric lymph (Vaerman *et al.*, 1997).

2.4. CONCLUSION

The gut-associated lymphoid tissue is organised in the inductive sites, the PP and the effector sites, the intestinal LP and epithelium. However, as discussed the distinctions between these inductive and effector sites are not absolute. Certain responses can be induced in the gut epithelium and LP, and effector cells might operate in the PP. For optimal vaccine development, it is crucial to know at which site in the gut the best immune response is induced with regard to the specific antigen/vaccine. Furthermore, as discussed in this review, sIgA plays an important role in the mucosal defence especially against extracellular pathogens, such as enterotoxigenic *Escherichia coli*. However, in addition to these sIgA, also IgM and to a lower extent IgG may provide mucosal protection.

PART II

AIMS OF THE STUDY

AIMS OF THE STUDY

Intestinal infections by enterotoxigenic *Escherichia coli* (ETEC) cause neonatal and postweaning diarrhoea in piglets. Neonatal diarrhoea can be prevented by passive lactogenic immunity achieved by parenteral immunisation of the sow. However, this passive protection disappears at weaning. In order to protect the newly weaned piglet against postweaning diarrhoea, an active immunity is needed. Hereto, parenteral vaccines are not ideal, since these vaccines stimulate the systemic rather than the mucosal immune system. Alternatively, oral vaccines should be used to stimulate the intestinal mucosal immune system.

In our laboratory it has been shown that oral administration of F4 (K88) fimbriae can protect weaned piglets against subsequent F4$^+$ETEC infection. However, it is not clear to what extent these F4 fimbriae are degraded in the stomach. Furthermore, knowledge on the cell type(s) which is (are) responsible for the translocation of the F4 fimbriae across the intestinal epithelial barrier and on the sites where the fimbriae activate the gut-associated immune system is lacking. Nonetheless, this knowledge is essential for the efficient development of a vaccine. Furthermore, information about the gastrointestinal transit and pH is necessary to develop effective oral delivery systems.

Therefore, the following questions were investigated:

- What are the gastrointestinal transit times of nondisintegrating pellets in suckling and recently weaned piglets?

- What is the gastrointestinal pH in suckling and recently weaned piglets? Are the F4 fimbriae degraded by the gastric pepsin or gastric and intestinal pH?

- Are the enterocytes or M cells responsible for the translocation of the F4 fimbriae across the intestinal epithelial barrier?

- At which sites in the gastrointestinal tract is the F4-specific immune response induced, and what is the major induction site: the lamina propria, the Peyer's patches of the jejunum or the ileal Peyer's patches?

- Can enteric coated pellets containing F4 fimbriae be used for oral vaccination against F4$^+$ETEC during the suckling period of the piglet as compared to F4 fimbriae in solution?

PART III

EXPERIMENTAL STUDIES

CHAPTER 3

GASTROINTESTINAL TRANSIT TIME OF NONDISINTEGRATING RADIO-OPAQUE PELLETS IN SUCKLING AND RECENTLY WEANED PIGLTES

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3.1. ABSTRACT

The objective was to determine the gastrointestinal (GI) transit times of pellets in piglets at different time points around weaning, as transit times are essential criteria to develop oral drug delivery systems. Nondisintegrating radio-opaque pellets were given orally in order to define the transit times by radiography. The radiographs were analysed with a software programme to calculate the number of pellets present in the different parts of the GI tract. In suckling piglets, the gastric emptying was faster (75% in 1.5 to 3.5 h) and the colonic accumulation (to 73%) was greater than in weaned piglets (3 days, 2 and 3 weeks postweaning, 65% gastric emptying in 18 h, 75% in 17 h, and 75% in 7 h, respectively; maximal colonic accumulations of 48%). Immediately after weaning, the transit was markedly prolonged but shortened with increased postweaning time (3 days, 2 and 3 weeks postweaning, 85% excretion in 175.5, 77, and 50.5 h, respectively). Three weeks postweaning, the transit was no longer affected by weaning as transit times were similar to values reported in growing and adult pigs, and retention appeared to be restricted to the stomach and the colon. These data are of crucial importance in the design of enteric coated formulations for oral administration of vaccines and therapeutics to young piglets and for human research using the pig model.

3.2. INTRODUCTION

Management of the newly weaned piglet presents one of the most significant challenges to swine producers. The stress of weaning and movement to another environment increases the risk for disease, especially diarrhoea, mainly caused by *enterotoxigenic E. coli* (ETEC) infections (Hampson, 1994; 1983; Richards and Fraser, 1961). These infections are commonly treated with antibiotics. However, the use of antibiotics may promote the development of resistant bacteria and increase the risk of contamination of meat products, causing concern for the consumer (Laval, 2000; Franco *et al.*, 1990; Dupont and Steele, 1987). Oral vaccination which protects the piglet to postweaning diarrhoea could provide an alternative handling of the problem (Van den Broeck *et al.*, 1999a; 1999b). However, vaccines and therapeutics are usually pH sensitive

biomaterials and should therefore be protected from the low pH in the stomach (O'Hagan, 1998).

Enteric coating of an oral drug formulation allows the release of the drug at the specific target site of the GI tract. Different enteric coating polymers are available, each dissolving above a specific pH (Lehman, 1994), and by varying the coat thickness the period over which the drug is protected against a specific pH can be adapted (Habib and Sakr, 1999). To select the appropriate coat thickness, knowledge on the gastrointestinal transit time of the formulation is required. Pellets are potential carrier systems for oral drug applications. As multiple unit systems, pellets give a more predictable gastric emptying time as compared to tablets or capsules: the evacuation through the pylorus is spread over a longer period of time and is less dependent on the state of nutrition as the pellets are sufficiently small to be evacuated during the digestive phase (Follonier and Doelker, 1992; Krämer and Blume, 1994). Moreover the inter- and intra-subject variability in bioavailability is lower due to the improvement in transit reproducibility and distribution along the digestive tract (Follonier and Doelker, 1992; Krämer and Blume, 1994). Pellets have the additional advantage that they are easily mixed with feed allowing a practical way of administration.

Although the pig has been considered as a valuable animal model (Kararli, 1995) for the evaluation of the GI absorption of new therapeutic agents and the performance of new dosage forms in humans (Hildebrand *et al.*, 1991; Kabanda *et al.*, 1994; Larsen *et al.*, 1992), only a few GI transit time data of nondisintegrating rigid oral dosage forms have been reported (Aoyagi *et al.*, 1992; Davis *et al.*, 2001; Hossain *et al.*, 1990). No reports are available on the transit time of pellets through the GI tract in suckling and recently weaned piglets, which are necessary to develop oral drug delivery systems for application in piglets at young age. The objective of this study was therefore to determine the GI transit times of nondisintegrating radio-opaque pellets using radiography in piglets at different time points around weaning.

3.3. MATERIAL AND METHODS

3.3.1. Pellet preparation

A pellet formulation was optimised which satisfied the following requirements. The pellets had to be nondisintegrating, sufficiently radio-opaque to be individually seen on the radiograph and have the ability to be mixed with feed allowing a convenient, stress-free method of administration. Low density polyethylene (PE), a powder of medical grade (ICOpolymers, Gravendeel, The Netherlands) and barium sulphate (Bufa Belgium, Brussels, Belgium) were used as components of the nondisintegrating radio-opaque pellets. Prior to hot stage extrusion, the different components of the pellet formulation were premixed in a Hobart A 200 planetary mixer (Kampenhout, Belgium). The mixture consisted of 40% low density PE and 60% barium sulphate. The extrusion was performed on an MP 19TC 25 laboratory scale co-rotating twin screw extruder of APV Baker (Newcastle-under-Lyme, UK) equipped with a circular die (diameter 2 mm) at a screw speed of 100 rpm, a powder feeding rate of 1.5 and a temperature of 130 °C. The extrudates were subsequently cut in pellets of 1 to 2 mm length by 0.5 to 1.5 mm diameter. The pellets had a density of 1.70 ± 0.01 g cm^{-3} (mean ± SD, n = 10). This density was calculated by dividing the sample mass by the sample volume. The sample volume was determined using He-pycnometry (Micromeretics, Norcross, GA, US).

3.3.2. Animals

Forty-four piglets of 5 litters (Large White x Piétrain) were used in the present study. Suckling piglets were housed in a room at 30 ± 3 °C. From 7 days of age onwards, they had free access to dry feed and water. Weaning was established at the age of 24-25 days. After weaning, they were housed in subgroups of 6 piglets in pens at 20 ± 3 °C with IR-lamps for local heating. The subgroups were made by randomly mixing different littermates. Weaned piglets had feed and water ad libitum. During the first 2 days postweaning, the animals received a standard starter meal (Baby-star, Quartes, Deinze, Belgium). The third day postweaning the feed was gradually switched to a standard grower diet (Pigo-star, Quartes, Deinze, Belgium). All animal pens (2.32 m^2) had reasonable space for free movement and normal activity of the piglets, thereby allowing

normal GI motility. The housing area was kept alight during day-time (12 h) and dark at night (12 h) and was close to the radiographic equipment. Experimental procedures and animal management procedures were undertaken in accordance with the requirements of the animal care and ethics committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2002/06).

3.3.3. Experimental set-up

Preliminary studies had revealed that handling of the piglets during imaging slowed down the transit. The gastric residence time doubled by analysing a piglet at 2, 4, 6, 8 and 12 h after administration of the pellets in comparison with analysing it once after 12 h. Therefore, the animals were randomly divided into different subgroups. Only 1 subgroup was examined at a time and the subgroups were alternated between the time spots. In doing so, the same subgroup was handled only once in a 12 h-period. The dose of pellets administered to the piglets, was optimised for each age group to clearly visualise the intestines.

3.3.3.1. Transit in suckling piglets

Eight littermates (21 days old, 4-6 kg) were used. Each piglet was given in the morning 10 cm^3 pellets mixed with water using an oral syringe. Subsequently the piglets were divided into 4 subgroups of 2 piglets. Radiographs were taken at the following hours postadministration from the subgroup mentioned in parentheses: 1.5 (1), 3.5 (2), 7 (3), 9.5 (4), 23.5 (1), 28.5 (2), 29.5 (3), 30.5 (4), 48 (1), 52.5 (2) and 53 (3) and this until the stomach, the small intestine and the caecum were completely emptied.

3.3.3.2. Transit in piglets 3 days postweaning

Thirty-six newly-weaned piglets (4 litters, 27-28 days old, 6-8 kg), housed in 6 subgroups of 6 piglets, were fasted for 12 h prior to administration of the pellets, but had water ad libitum. Per 6 piglets, 72 cm^3 pellets were mixed with meal to a total volume of 300 cm^3. This pellet/meal mixture was divided over 3 bowls, allowing easy access for all piglets within the subgroup. The pellet/meal mixture was administered in the morning and was consumed within 30 min. Afterwards the piglets obtained feed and water ad libitum. Radiographs were taken at the following hours postadministration, from the

subgroup mentioned in parentheses: 4 (1), 6 (2), 8 (3), 10 (4), 12 (5), 14 (6), 16 (1), 18 (2), 25 (3), 26 (4), 28 (5), 30 (6), 32 (1), 34 (2), 54.5 (3). Later on, radiographs were taken far less frequently, and at 104, 126.5, 151.5, 175.5 and 200 h postadministration, a new subgroup of 6 piglets, composed by taking randomly one piglet of each subgroup, was monitored. This new subgroup represented the initial 6 subgroups. Radiographs were taken until 90 % of the pellets was excreted. Analysis of the radiographs demonstrated that 8 piglets had eaten an insufficient amount of pellets, making it impossible to localise the pellets with certainty. Therefore the data of these animals were withdrawn from the study.

3.3.3.3. Transit in piglets 2 weeks postweaning

The same 36 piglets (38-39 days old, 9-11 kg), as studied 3 days postweaning, were used. The piglets of 5 subgroups (30 piglets) were given the pellet/meal mixture in the morning and radiographs were taken at the following hours postadministration from the subgroup mentioned in parentheses: 3 (1), 6 (2), 9 (3), 12 (4), 15 (5), 17 (1), 24.5 (2), 28.5 (3), 32 (4), 49.5 (5), 53.5 (1), 54.5 (2), 72.5 (3), 77 (4). Radiographs were taken until more than 95 % of the pellets was excreted. Subgroup 6 (6 piglets) was given the pellet/meal mixture in the evening and radiographs were taken at 14, 16 and 38.5 h postadministration to assess the influence of the night on the GI transit time.

3.3.3.4. Transit in piglets 3 weeks postweaning

In this experiment only one subgroup of 6 piglets (45-46 days old, 10-13 kg) was used. The pellet/meal mixture was administered in the morning and radiographs were taken 7, 25, 31, 50.5 and 74.5 h postadministration. Since the GI transit was considerably faster than in the piglets 2 weeks postweaning, an additional measurement was performed at 31 h postadministration. Since only 1 subgroup was used, the 12 h interval between the successive radiographic measurements could not be maintained. This had apparently no influence on the GI transit time, as will be discussed later.

3.3.4. Radiographic analysis

Radiography was used to study the passage of nondisintegrating radio-opaque pellets through the GI tract. It is important to note that the piglets were not sedated, since

sedation and anaesthesia slow down the transit time in the pig (Nimmo, 1989). Radiographic examinations were performed with a Europa 3TS radiographic instrument (X-Ray Equipement Verachtert BVBA, Antwerpen, Belgium). Super HR-E 30 Fuji Medical X-ray film with rare earth screens (Fuji G12) were exposed at focus-to-film-distance of about 80 cm. Exposure settings were as follows: for suckling piglets 57 kV and 3.2 mAs and for piglets postweaning 61 kV and 3 mAs. The piglets were manually restrained during imaging. Laterolateral and ventrodorsal views were taken to allow a precise localisation of the pellets and to exclude any false interpretations of the localisation due to superposition of different parts of the intestine. The time needed to reposition the piglets was approximately 30 s.

The radiographs were photographed with a digital camera Leica Digilux 4.3 (Leica Camera AG, Solms, Germany). These pictures were analysed with the software programme Optimas® version 6.5 (Media Cybernetics L.P., Silver Spring, USA). Regions of interest (ROI) that corresponded to the stomach, the small intestine, the caecum, and the colon were outlined as shown in Fig. 3.1. Due to the convoluted nature of the GI tract of the pig, further subdivisions of the intestinal tract were not attempted, as this could not be done accurately in every radiograph.

Each picture was analysed as follows: in the different ROI, pellets, which were individually discernible, were counted. The number of pellets in a spot was calculated based on the number of pixels in the spot and the intensity of the spot to correct for superposition of different pellets using the following equation: pellets calculated in spot=(pixels in spot/pixels per pellet) x (intensity spot/intensity pellet).
Summation of the pellets counted and calculated (equation) yielded the total number of pellets per ROI.

The radiographs taken immediately postadministration were used to determine the number of pellets ingested, which is the sum of the amount of pellets in the different ROI. For each subsequent image, the number of pellets was expressed as a percentage of the number ingested. The percentage of pellets, present in the different parts of the GI tract on the laterolateral and the ventrodorsal image, was averaged and this number was used

for further analysis. Sites of accumulation were defined as sites where the pellets are retained for a longer period of time and where the amount of pellets is increased with increasing time postadministration.

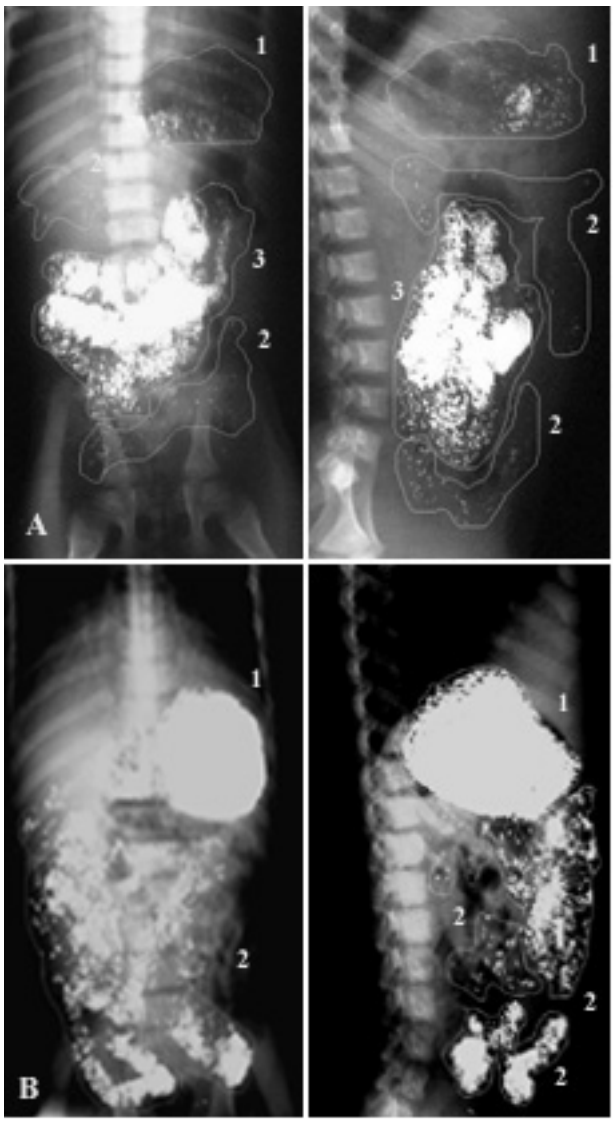


Fig. 3.1. Examples of different regions of interest used for analysis of the radiographs on dorsoventral (left) and lateral (right) views of a suckling piglet 9.5 h post pellet administration (A) and of a 3 days-weaned piglet 4 h post pellet administration (B) are shown; (1) the stomach, (2) the small intestine and (3) the colon.

3.3.5. Statistics

The stomach emptied exponentially and the regression ($y=ae^{bx}$) could be semi-logarithmically transformed to produce a linear regression of the form $\mathrm{Ln}(y)= \mathrm{Ln}(a) + bx$, where y is the fraction of ingested pellets (%) present in the stomach, x the time postadministration in hours, a is a constant and b is the rate constant. For each pig the rate

constant was calculated and these values were used to compare gastric emptying rates by analysis of variance.

The faecal excretion of the pellets also occurred exponentially and followed a curve of the form $y=b\text{Ln}(x)+a$, where y is the fraction of the ingested pellets (%) that is excreted, x is the time postadministration in hours, a is a constant and b is the rate constant. The onset of the faecal excretion is given by the x value when the y is zero in the equation. For each pig the rate constant and the onset of faecal excretion was calculated and these values were used to compare overall transit by analysis of variance.

Statistical analysis was performed using the software package SPSS version 11.0. The data (rate constants and onset of faecal excretion) were tested for normal distribution with a Kolmogorov-Smirnov test and were non-normally distributed. Differences in gastric emptying and overall transit time between the different parameters were tested for statistical significance using the Mann-Whitney U test. The parameters tested were: (a) differences between the different age groups (groups to each other), (b) differences in gastric emptying between the different subgroups (subgroups to each other) of the 3 days postweaning group, (c) difference between administration of the pellets in the morning (all five subgroups) or in the evening (one subgroup) two weeks postweaning. The significance level was set at 5 %.

3.4. RESULTS

Data on the GI transit time at different ages and in the different parts of the GI tract i.e. the stomach, the small intestine, the caecum, the colon, and the total excretion are shown in Fig.3.2A, 3.2B, 3.2C, 3.2D and 3.2E, respectively. A summary is shown in Table I.

3.4.1. Transit in suckling piglets

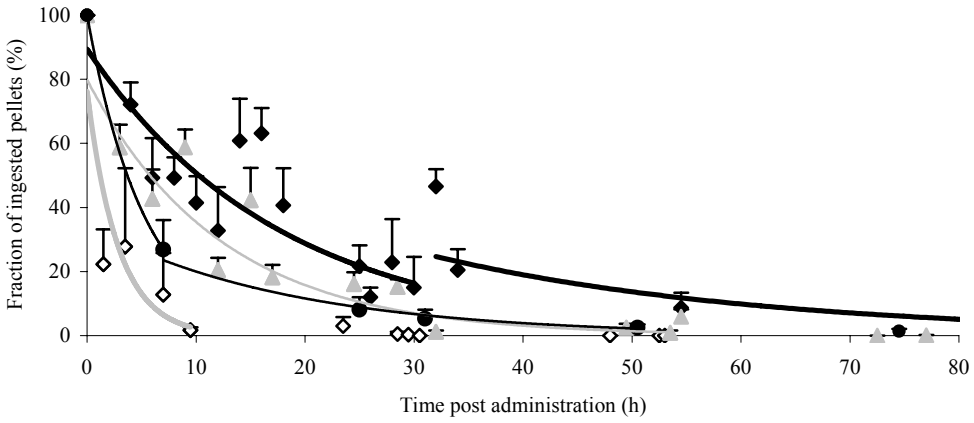
The stomach emptied exponentially. The data [mean fraction of the ingested pellets (%) present in the stomach] followed a curve with the equation $y = 76.216e^{-0.325x}$ (R^2= 0.853). Seventy-five % of the pellets left the stomach during the first 1.5 to 3.5 h. The stomach was emptied for more than 98% after 9.5 h. The remaining 2% were evacuated during the next 20 h. The pellets passed exponentially through the small intestine. The

data [mean fraction of ingested pellets (%) present in the small intestine] fitted the curve $y = 86.372e^{-0.0845x}$ (R^2= 0.840). It lasted for more than 9.5 h before more than 75% of the pellets left the small intestine. There was no accumulation in the caecum. However, there was retention in the colon, reaching accumulations of 53 to 73% between 9.5 and 30.5 h postadministration. The pellets first appeared in the faeces after 23.5 h and were exponentially excreted following the equation $y = 61.501\text{Ln}(x) - 191.06$ (R^2= 0.837). Fifty % was excreted after 53 h.

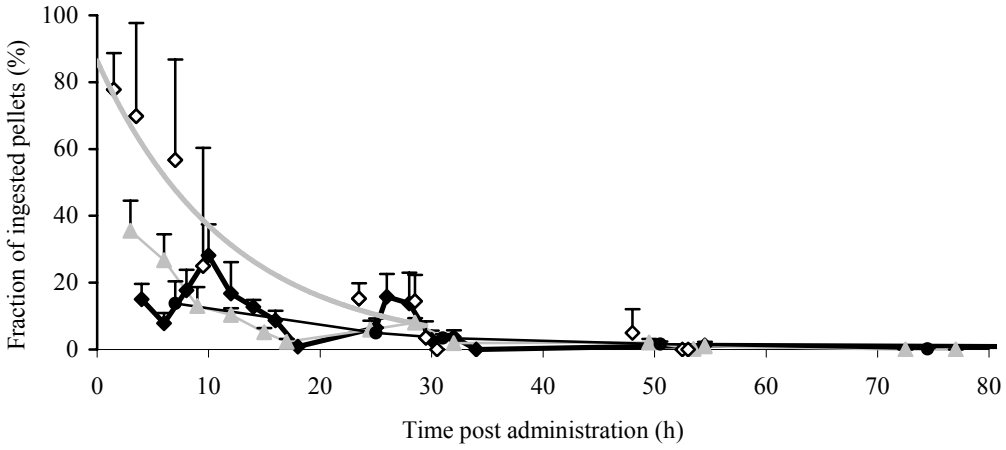
3.4.2. Transit in piglets 3 days postweaning

The gastric emptying was faster during the first 30 h than later on. Untill 30 h postadministration, the data [mean fraction of ingested pellets (%) present in the stomach] followed the exponential curve $y = 89.393e^{-0.0566x}$ (R^2= 0.765), whereas the data from 32 to 200 h postadministration fitted the exponential curve $y = 70.518e^{-0.0328x}$ (R^2= 0.972). The first regression equation yielded a R^2 value of 0.765. Variation in gastric emptying between the different subgroups, which were used to study the transit in this age group, was observed and caused this lower R^2 value. However, no significant difference in gastric emptying between the different subgroups was found. Sixty-five % of the pellets left the stomach after 18 h. It was only after 54.5 h that 90% of the stomach was emptied, and traces were present until 200 h postadministration. No accumulation of the pellets was noticed in the small intestine. However, retention was found in the caecum (accumulation of 41 % was noticed) and especially in the colon (32 to 48% between 25 and 54.5 h postadministration). Faecal excretion followed a curve with the equation $y = 47.48\text{Ln}(x) - 159.77$ (R^2= 0.987). Faecal excretion started after 30 h, while 70 and 85 % were excreted after 126.5 and 175.5 h, respectively.

(A) Stomach



(B) Small Intestine



(C) Caecum

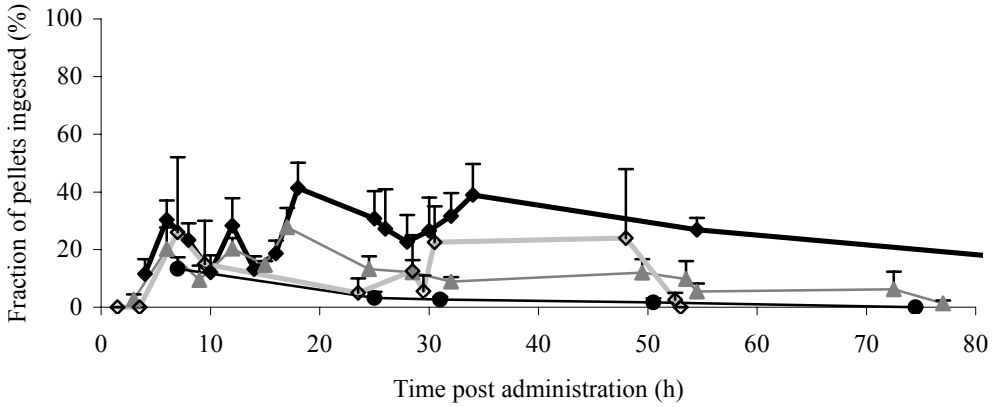
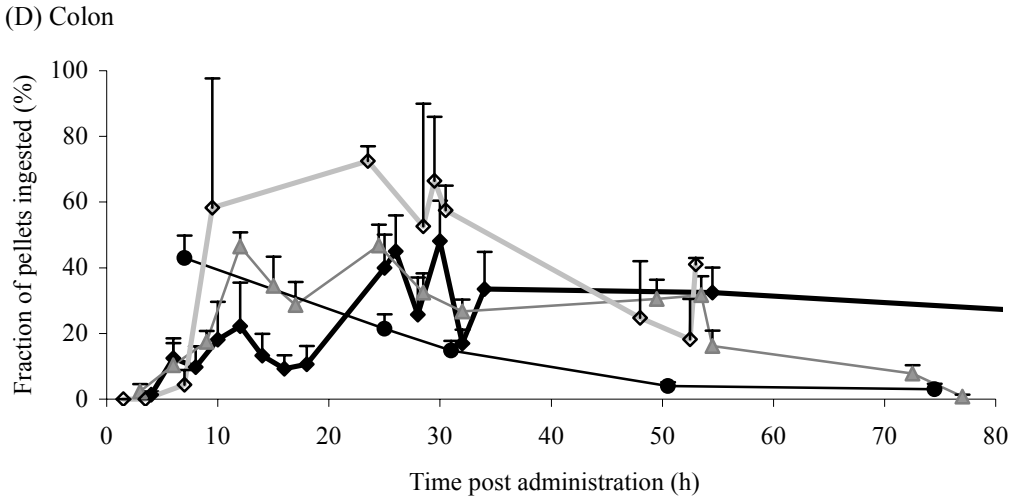


Fig. 3.2. Fraction of ingested pellets (%) present in the stomach (A), the small intestine (B), the caecum (C), the colon (D), and fraction of the ingested pellets (%) excreted in the faeces (E) of the suckling piglet (mean ± SD, n = 2, —◊—), the piglet 3 days (mean ± SEM, n = 3-6, —♦—), 2 weeks (mean ± SEM, n = 6, —▲—) and 3 weeks postweaning (mean ± SEM, n = 6, —●—) at different hours postadministration.



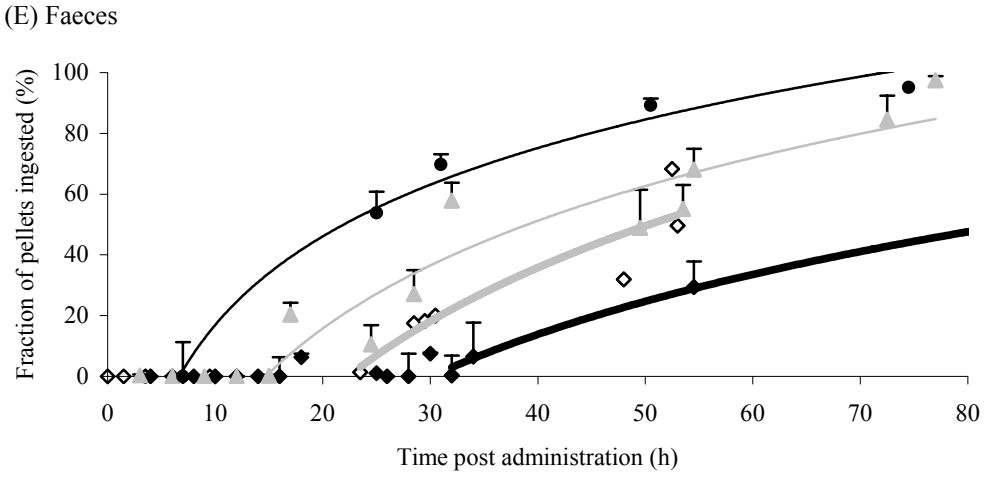


Fig. 3.2. (continued). Fraction of ingested pellets (%) present in the stomach (A), the small intestine (B), the caecum (C), the colon (D), and fraction of the ingested pellets (%) excreted in the faeces (E) of the suckling piglet (mean ± SD, n = 2, —◊—), the piglet 3 days (mean ± SEM, n = 3-6, ━♦━), 2 weeks (mean ± SEM, n = 6, —▲—) and 3 weeks postweaning (mean ± SEM, n = 6, —●—) at different hours postadministration

3.4.3. Transit in piglets 2 weeks postweaning

The gastric emptying occurred exponentially. The data [mean fraction of pellets (%) present in the stomach] fitted the curve $y = 79.814e^{-0.0811x}$ ($R^2 = 0.837$). The gastric emptying was rapid during the first 17 h (75%), followed by a gradual loss of the pellets from the stomach over the next 38 h, with already less than 5 % retained after 32 h. There was no accumulation in the small intestine and in the caecum. On the other hand, the pellets accumulated in the colon which retained 30% to 46% of the pellets between 12

and 53.5 h. Faecal excretion followed a curve with the equation y =50.831Ln(x)-136.19 (R^2= 0.816). Faecal excretion started after 15 h and after 72.5 and 77 h, 80 and 85% were excreted, respectively.

No significant difference in gastric emptying and overall transit was found between administration of the pellets in the morning and in the evening (data not shown).

3.4.4. Transit in piglets 3 weeks postweaning

The gastric emptying was fast during the first 7 h as the stomach was already emptied for 75%. Since the first measurement was performed at 7 h, it could not be concluded if this emptying was exponential (y =100e$^{-0.1875x}$) or linear (y =-10.44x + 100). From 7 h onwards, the emptying slowed down and the data fitted the exponential curve y =34.47e$^{-0.0544x}$ (R^2= 0.974). In contrast with the small intestine and the caecum, the pellets were retained in the colon, which contained 43 % of the pellets after 7 h. From 25 h postadministration on, no further accumulation was noticed. There was a gradual loss of pellets from the colon over the next 68 h, but after 50.5 h only 4 % were retained. Faecal excretion followed the curve y =42.011Ln(x)-79.794 (R^2= 0.985). The pellets first appeared in the faeces from 7 h onwards and 55 and 85 % were excreted after 25 and 50.5 h, respectively.

3.4.5. Comparison between the different age groups

The gastric emptying in suckling piglets was significantly faster than in piglets 3 days ($P<0.05$) and 2 weeks postweaning ($P<0.05$), and faster than 3 weeks postweaning ($P=0.06$). Three days postweaning the gastric emptying was slower than 2 weeks ($P=0.06$) and significantly slower than 3 weeks postweaning ($P<0.05$) (Fig. 3.2A).

No significant differences were found between the rate constants for the faecal excretion of the different age groups (Fig. 3.2E), which is demonstrated by the parallel course of the faecal excretion curves. However, the onset of faecal excretion differed significantly. Three weeks postweaning the onset was significantly earlier than for the other age groups ($P<0.05$). Two weeks postweaning the onset was significantly earlier than for the suckling piglets ($P<0.05$) and piglets 3 days postweaning ($P<0.05$), whereas no difference was found between the suckling piglets and piglets 3 days postweaning.

Comparison of the gastric empyting curves with the faecal excretion curves demonstrated that the faster the gastric emptying, the faster the onset of the faecal excretion was. However, whereas the gastric emptying in the suckling piglets is faster compared to the piglets postweaning, this is not the case for the onset of the faecal excretion.

Table 1: Summary of GI transit time of pellets in suckling and recently weaned piglets.

Pellets Present (%)	Suckling Piglet (h)	3 Days postweaning (h)	2 Weeks postweaning (h)	3 Weeks postweaning (h)
Stomach				
75	ND	4 – 6	<3	ND
50	ND	8 – 16	6 – 9	ND
25	1.5 – 3.5	25 – 34	12 – 17	7
10	<7	54.5	30	<25
<5	<9.5	104	32	31
Small intestine				
75	1.5 – 3.5 h	-	-	-
50	7	-	-	-
25	9.5	-	6	-
10	28.5	(4-28 h)[a]	12	>7
<5	30.5	30	15	<25
Caecum				
25 – 40[b]	-	6 – 54.5	-	-
Colon				
30 – 50[c]	-	25 – 54.5	12 –53.5	7
50 – 75[d]	9.5 – 30.5	-	-	-
Faeces				
1	23.5	30	15	7
85	>53	175.5	77	50.5

ND, not determined
-: not detected

[a]Varying form 10% to 20%.
[b]Accumulations detected in the caecum varied from 25 to 40%.
[c]Accumulations detected in the colon varied from 30 to 50%.
[d]Accumulations detected in the colon varied from 50 to 75%.

3.5. DISCUSSION

The pellets used in this study had a density of 1.7 g cm^{-3}. In humans there may be a threshold density of 2.4 to 2.6 $g.cm^{-3}$ above which the gastric emptying is prolonged (Clarke *et al.*, 1993). However, Davis *et al.* (1986) conclude that the human gastric emptying of solid dosage forms depends mainly on the size of the dosage form and on the presence of food in the stomach and not on the density. No reports are available on the relation between gastric residence time and pellet density in the pig. The present study indicated a large variation in the gastric residence time, the overall transit time and the sites of pellet retention between suckling and weaned piglets on one hand and with the time postweaning on the other hand.

In the suckling piglet, mainly fed by the sow's milk, the total gastric residence time is shorter than in the weaned piglet on a dry diet. This can be explained by the fact that liquids empty much faster than solids (Camilleri *et al.*, 1985; Gregory *et al.*, 1990). In suckling piglets, the liquid diet carries the pellets, resulting in a faster gastric emptying, whereas in the weaned piglet the pellets are mixed with the dry diet, which is emptied slower. The gastric emptying times are in agreement with those reported by Kidder *et al.* (1961; 1968) and Kvasnitskii (1951), who found in the suckling piglet gastric emptying times for feed ranging from 1.5 to 2 h, although traces were detected in the stomach until 7.5 h postadministration. More pellets accumulate in the colon as compared to the weaned piglet. This is probably due to the faster gastric emptying so that the major fraction of pellets enters the colon within a shorter period and a slower colonic transit. The lower fibre content of the liquid diet may account for a slower colonic transit as fibre increases the rate of passage through the large intestine (Potkins *et al.*, 1991). As a consequence, only after 23 h the first pellets appeared in the faeces, which is a lot later than in the 3 weeks-weaned piglet (7 h) in spite of the faster gastric emptying. A similar observation was made by Kidder *et al.* (1961; 1968).

Three days postweaning, the transit time is markedly prolonged and an accumulation of the pellets is seen in almost every part of the GI tract. This is probably due to the weaning, which is extremely stressful. Social stress may result from removal of

the sow, relocation to a new environment and introduction to strange penmates so that a new social structure has to be established (Hessing *et al.*, 1994; Meese and Ewbank, 1973). Nutritional stress may occur due to the change from a liquid to a dry diet and leads to a reduced feed intake during the first days postweaning (Lecce *et al.*, 1979). Physiological stress is caused by a series of changes in the small intestinal structure and the enterocyte brush border enzyme activities immediately after weaning, which are responsible for a reduction in intestinal digestive and absorptive function (Kenworthy, 1976; Pluske *et al.*, 1997; Tang *et al.*, 1999; Xu *et al.*, 2000). Environmental stress may occur when newly weaned pigs are taken from a warm farrowing house to a nursery with colder temperatures (LeDividich *et al.*, 1980). All these stress factors have an enormous influence on the GI motility, explaining the long transit times observed 3 days postweaning. A non-significant variation in transit was found between the different subgroups at this age, which caused the lower R^2 value for the gastric emptying curve. This can be attributed to the fact that a stable social structure is not developed as fast in every group and to differences in stress sensibilities of the individual piglets (Hessing *et al.*, 1994; Giroux *et al.*, 2000).

Two weeks postweaning, the transit time is considerably accelerated compared to 3 days postweaning. The gastric emptying is more rapid, there is no apparent accumulation in the caecum, the retention in the colon is lower and consequently the overall transit time is shorter.

Three weeks postweaning, the GI transit seems no longer affected by the stress of weaning. The present transit times agree to values previously reported for growing and mature pigs. The transit data at 3 weeks postweaning indicate an initially rapid emptying of the stomach as observed for the emptying of feed and pellets from the mature pig's stomach (Gregory *et al.*, 1990; Potkins *et al.*, 1991; Johansen *et al.*, 1996; Davis *et al.*, 2001; Guerin *et al.*, 2001). These authors reported 2.5 to 6 h for 50 % stomach clearance, depending on the body weight, the nature of the diet, the feeding regime, the density and the size of the feed markers or pellets. The rate of gastric emptying increases with body weight, which suggests that the rate of emptying may be linked in one way or another

with the metabolic requirements of the body (Gregory *et al.*, 1990). Other factors which can be involved are an increased muscularity of the stomach, an increased pyloric diameter and an increased duodenal capacity with increase in size of the animal. Feeding postpones the activity of the migrating myoelectric complex (MMC) which prolongs the gastric residence time (Itoh *et al.*, 1986; Aoyagi *et al.*, 1992; Hossain *et al.*, 1990). Our data also demonstrate that pellets can spend a very long time in the stomach, e.g. in the 3 weeks-weaned piglets, the stomach is not completely emptied even after 50.5 h. This is in agreement with findings on the feed passage in the mature pig (Neimeier, 1940; Kvasnitskii, 1951; Clemens *et al.*, 1975). Furthermore the transit is relatively rapid through the small intestine and the caecum, whereas prolonged retention is noticed in the colon. Clear caecal accumulation of the pellets is only observed in the 3 days-weaned piglets. This indicates that the role of the caecum as a site for prolonged feed/pellet retention is limited. In all piglets colonic retention indicates that the large intestine is the primary organ regulating the rate of passage for ingested pellets. From the present data it is apparent that ingested materials are retained in the stomach and in the colon. This conforms well to the findings of Clemens *et al.* (1975) for solid feed markers in the mature pig.

Although Kumar *et al.* (1986) found a significant reduction in small bowel transit at night in mature pigs, our piglets did not differ significantly in gastric emptying and overall transit time between night and day.

3.6. CONCLUSION

In suckling piglets, the gastric emptying is faster and the colonic accumulation is greater than in weaned piglets. Weaning is an extremely stressful situation which accounts for prolonged transit times the first weeks postweaning. Three weeks postweaning, the transit is no longer affected by weaning and retention of the pellets is only restricted to the stomach and the colon, having retention times comparable to those of growing and adult pigs as reported in the literature. The variation in transit times at different time points around weaning should be taken into account when designing an

enteric coating for oral administration of vaccines or therapeutics to suckling and recently weaned piglets and when using the pig model for human research.

3.7. ACKNOWLEDGEMENTS

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CHAPTER 4

INFLUENCE OF PORCINE INTESTINAL pH AND GASTRIC DIGESTION ON ANTIGENICITY OF F4 FIMBRIAE FOR ORAL IMMUNISATION

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4.1. ABSTRACT

Newly weaned piglets can be orally immunised against F4+ *enterotoxigenic Esherichia coli* (ETEC) infection with F4 (K88) fimbriae. However, to efficiently develop a vaccine against ETEC induced postweaning diarrhoea, knowledge of the stability of the F4 fimbriae to different pH and gastric digestion is needed. The gastrointestinal pH in suckling and recently weaned piglets was measured and the stability of F4 fimbriae to different pH and to pepsin was assessed *in vitro*. In the stomach the lowest pH was found in the fundus gland region. Gastric pH values below 2.5 were not found in suckling piglets or at the day of weaning, in contrast to piglets 1 and 2 weeks postweaning. Along the first half of the small intestine and in the caecum, a negative correlation was found between pH and age. The F4 fimbriae were stable to pH 1.5 and 2 for 2 hours, whereas longer incubation periods resulted in conversion of the multimeric forms into monomers. The F4 fimbriae were partially degraded by incubation for 15-30 min in simulated gastric fluid at pH 1.5 and 2, and completely digested from 3 hours onwards. At pH 3, the fimbriae maintained their antigenicity for at least 4 hours. The results demonstrate that gastric digestion will only have a limited impact on oral immunisation since liquid passes through the stomach relatively quickly (50 % within 2 hours). However, we previously demonstrated that the transit times are prolonged shortly after weaning. Shortly after weaning it could be necessary to protect the F4 fimbriae against gastric digestion to obtain efficient oral immunisation of the piglets.

4.2. INTRODUCTION

F4$^+$ *enterotoxigenic E. coli* (ETEC) are a major cause of postweaning diarrhoea in piglets, which is responsible for severe economic losses due to both mortality and reduced growth rates (Hampson, 1994). The F4 fimbriae allow the ETEC to adhere to F4-specific receptors (F4R) on brush borders of villous enterocytes. They are long proteinaceous appendages radiating from the surface of the ETEC with a length of 0.1-1 µm and a diameter of 2.1 nm (Stirm *et al.*, 1967) and are composed of several hundred mainly identical protein subunits (Klemm, 1981), called FaeG, as well as some minor subunits. Van den Broeck *et al.* (1999a) demonstrated that newly weaned piglets can be orally

immunised against $F4^+$ ETEC infections with F4 fimbriae in solution. The ability of the F4 fimbriae to bind to the receptor on the brush border of the small intestinal villous enterocyte is however a prerequisite for their oral antigenicity. Low pH and proteases can break down proteins. Therefore resistance of the F4 fimbriae to gastric pH and digestion by pepsin is crucial.

In this study, the pH along the GI tract was studied in piglets at different time points around weaning. Thereafter the stability of the F4 fimbriae to the determined gastrointestinal pH and to pepsin was evaluated *in vitro*.

4.3. MATERIAL AND METHODS

4.3.1. GI pH measurement

Twenty-four piglets of 4 litters (Large White x Piétrain) were used. Suckling piglets were together with the sows housed in a pen at 30 ± 3 °C. From 7 days of age onwards, they had free access to the starter meal (Baby-star, Quartes, Deinze, Belgium) and water. Piglets were weaned at the age of 24 days and were housed in isolation units at 20 ± 3 °C with IR-lamps for local heating and fed ad libitum (standard grower diet, Pigo-star, Quartes, Deinze, Belgium). The GI pH (n=6) was measured in suckling piglets (17 days old) at the day of weaning (24 days old) and 1 week and 2 weeks postweaning (30 and 38 days old, respectively). Piglets were euthanised by intravenous injection of an overdose of pentobarbital (24 mg/kg; Nembutal, Sanofi Santé Animale, Brussels, Belgium). Special care was taken to prevent mixing of gastric and intestinal contents by moving the pigs as less as possible (Regina *et al.*, 1999). Immediately after slaughter, the GI tract was removed. In all animals, the pH was measured *in situ* in the fundic glandular region of the stomach, the small intestine, the caecum, the colon and the rectum. In the small intestine the pH was measured at fixed points namely 1 cm distal from the pylorus, and at 1/16, 1/8, 1/4, 1/2, 3/4 and 4/4 of the length of the small intestine. The first 2 points (1 cm distal from the pylorus and 1/16) correspond to the duodenum, the last point to the terminal ileum. The other points were located in the jejunum. In the stomach of suckling piglets, the pH was additionally measured in the diverticulum, the cardiac, the fundus and the pyloric gland regions and close to the torus pyloricus. All pH measurements were

performed by insertion of a pH probe into the lumen via a small incision through the gut wall (portable Sentron pH meter type Argus with Lancefet probe, Sentron Europe B.V., Roden, The Netherlands).

Experimental procedures and animal management procedures were undertaken in accordance with the requirements of the animal care and ethics committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2002/06).

4.3.2. Purification of F4 fimbriae

The F4 fimbriae were purified as described by Van den Broeck et al (1999c). Briefly, fimbriae were isolated by homogenizing the bacterial suspension of E. *coli* strain GIS 26 (O149:K91:F4ac, LT^+, STa^+, STb^+) using an Ultra Turrax (Janke & Kunkel, IKA Labortechnik, Staufen, Germany). The solubilised fimbriae were subsequently precipitated with 40 % (w/v) ammonium sulphate, whereafter the pellet was dissolved and dialysed overnight against PBS. The protein concentration of the fimbrial preparation was determined using the Bicinchoninic Acid Protein Assay Kit (SIGMA, Sigma-Aldrich, Bornem, Belgium).

4.3.3. Incubation at different pH

The F4 fimbrial solution (518 µg / ml in 200 µl PBS) was adjusted to different pH (1.5, 2, 3, 4 or 7) with 1 M HCl and incubated during 1, 2, 2.5 and 3.5 hours at 37 °C under gentle rotation at 80 rpm. Subsequently, the samples were neutralised (pH 8) by adding 60 µl of 200 mM Na_2CO_3. Fifty-one µl SDS-PAGE sample loading buffer (30% glycerol, 15 % β-mercaptoethanol, 9% SDS, 180 mM Tris [pH 6.8], and 0.02 % bromophenol blue) was added to the samples and 15 µl of each sample was loaded on the gel.

4.3.4. Incubation in simulated gastric fluid

Simulated gastric fluid (SGF) (United States Pharmacopeia, 1995) contained 0.32 % (w/v) pepsin (SIGMA, Sigma-Aldrich, Bornem, Belgium) in 0.03 M NaCl solution at pH 1.5, 2, 3, 4 and 7 as control. Proteolytic activity of SGF preparations was estimated by measuring trichloroacetic acid precipitable haemoglobin (SIGMA) after hydrolysis for 10 min as described by Ryle (1984). Incubations in SGF were performed as described by

Fuchs *et al.* (1993). Briefly, 16 ng/µl F4 protein was digested in 200 µl aliquots of SGF in eppendorf tubes preheated to 37°C. Incubations were maintained at 37°C at 80 rpm and quenched by neutralisation (pH 8) with 60 µl of 200 mM Na_2CO_3 after 15 and 30 min and 1, 2, 3, 4 h. Fifty-one µl SDS-PAGE sample loading buffer as described in section 2.3 was added and 20 µl of each sample was loaded on the gel.

4.3.5. SDS-PAGE and Immunoblotting

Degradation or digestion of the multimeric F4 fimbriae was evaluated by SDS-PAGE (12 %) without previously boiling the samples. Proteins were visualised by Coomassie blue or silver staining (Harlow and Lane, 1988). Relative molecular weight protein markers (250 – 10 kDa) (Precision Plus Protein Standard, Bio-Rad Laboratories, Hemel Hempstead, Herts, United Kingdom) were run with each gel. The gels were blotted onto 0.45 µm polyvinylidene fluoride membranes (Gelman Sciences, Vel, Leuven, Belgium) by semi-dry electrotransfer (Kyhse-Andersen, 1984). The blots were developed with the F4 specific MAb (IMM01, Van der Stede *et al.*, unpublished data), a rabbit anti-mouse horseradish peroxidase conjugate (DAKO, Glostrup, Denmark) and aminoethylcarbazole (SIGMA) and H_2O_2 as substrate for the enzymatic reaction as described (Van den Broeck *et al.*, 1999c).

4.3.6. Statistical analysis

The pH data were tested for normal distribution with a Kolmogorov-Smirnov test and the homogeneity of variances was tested with a Levene's test. Statistical analysis was performed using the software package SPSS version 9.0. All parameters were assessed for statistical significance using one way ANOVA. The effects of the parameters were further compared by performing a multiple comparison among pairs of means, using a Bonferroni test. Statistical significance was defined as $P < 0.05$. The relationship between the GI pH and age or the distance from the stomach, was estimated by using the Pearson's correlation test ($P < 0.01$).

4.4. RESULTS

4.4.1. Gastric pH in suckling and recently weaned piglets

In suckling piglets the pH was measured in different parts of the stomach (Fig. 4.1). The pH in the diverticulum and close to the torus pyloricus (3.9 ± 0.9 and 4.1 ± 0.1, respectively) were significantly higher than in the fundus gland region (3.0 ± 0.3).

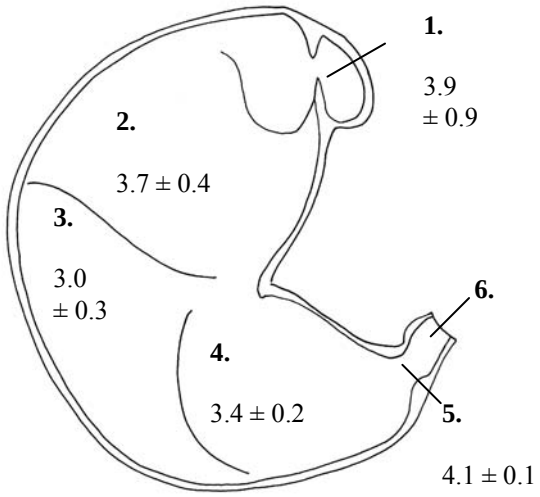


Fig. 4.1. Gastric pH (mean ± SD, n = 6) in different parts of the stomach in the suckling piglet. Frontal section of the pig's stomach: 1. Diverticulum, 2. Cardiac gland region, 3. Fundus gland region, 4. Pylorus gland region, 5. Torus pyloricus, 6. Duodenum.

The mean pH in the fundus gland region at different ages is shown in Fig. 4.2. No significant differences could be found in this gastric pH between piglets of different ages. In the weaned piglets, the gastric pH ranged from 2.7 to 4.7 at the moment of weaning, from 1.6 to 4.4 one week postweaning, and from 1.7 to 3.9 two weeks postweaning.

4.4.2. Small intestinal pH in suckling and recently weaned piglets

The pH values at any point along the small intestine varied less than the values in the stomach, except for the pH at 1 cm distal from the pylorus (Fig. 4.2). There, the pH varied between 5.1 and 6.5 in the suckling piglets, between 4.2 and 6.1 at the moment of weaning, between 3.2 and 5.8 one week postweaning, and between 2.4 and 6 two weeks postweaning. From 1/16 of the length of the small intestine onwards, pH values lower than 4.6 were never found. The pH increased with increasing distance from the stomach in all groups. This implicated that the pH and the distance from the stomach were

positively correlated: the correlation coefficient (r) was 0.585 ($P < 0.01$) for suckling piglets, 0.729 ($P < 0.01$) for piglets at the moment of weaning, 0.741 ($P < 0.01$) for piglets 1 week postweaning and 0.731 ($P < 0.01$) for piglets 2 weeks postweaning. However, at 3/4 (distal jejunum) and 4/4 (ileum) of the small intestine the pH remained stable. Here, maximum pH levels were found namely 6.5 in the suckling piglets, 6.8 at the day of weaning, 6.8 at 1 and 6.7 at 2 weeks postweaning. In the suckling piglets the change in pH along the small intestine was rather small (lower correlation coefficient) and in contrast to the other groups, individual pH values as high as 7 were never found.

The pH along the first half of the small intestine did decrease significantly with age (r ($P < 0.01$) were -0.558, –0.733, -0.773 and -0.779 at 1 cm from the pylorus, 1/16, 1/8, and 1/4 of the length of the small intestine, respectively). This was not the case for the caudal half (1/2, 3/4, and 4/4) of the small intestine.

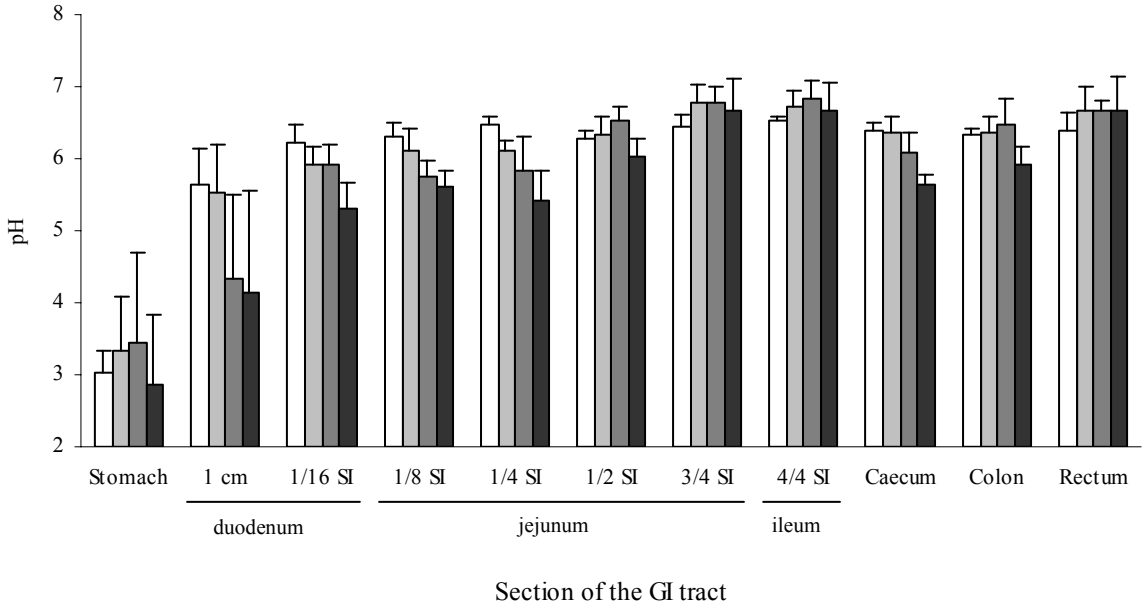


Fig. 4.2. pH (mean ± SD, n = 6) of GI contents of the suckling piglet (□), the piglet at the day of weaning (■), 1 (■) and 2 weeks postweaning (■). The pH was measured in the stomach in the fundus gland region and at fixed points along the small intestine namely 1 cm (distal from the pylorus) and 1/16, 1/8, 1/ 4, 1/2, 3/4, 4/4 of the length of the small intestine.

4.4.3. Large intestinal pH in suckling and recently weaned piglets

In the caecum, the pH decreased to 6.4 in the suckling piglets, 6.4 in the piglets at the moment of weaning, 6.1 in the piglets 1 week postweaning and 5.6 in piglets two weeks postweaning (Fig. 4.2). This decrease was significant for all age groups except for the suckling piglet. Furthermore there was a negative correlation ($r = -0.791$, $P < 0.01$) between the pH in the caecum and the age.

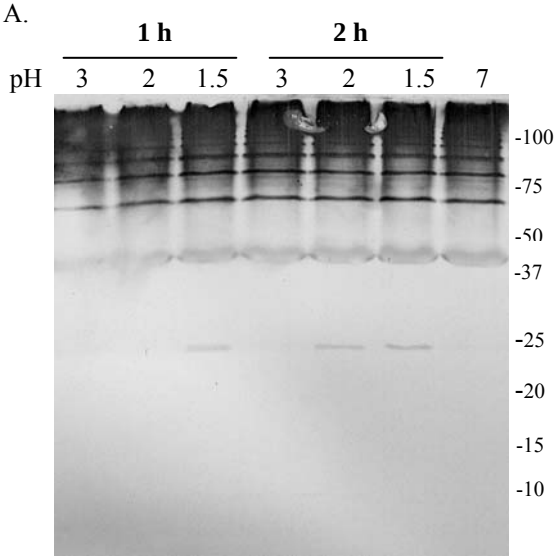
In the colon (range 5.9-6.5) and the rectum (range 6.4-6.7), pH did not change significantly from the caecal pH nor did it change significantly with age (Fig. 4.2).

4.4.4. Degradation of the F4 fimbriae at different pH

Degradation of the multimeric F4 fimbriae by incubation at pH between 1.5 and 7 for periods of 1, 2, 2.5 and 3.5 hours was evaluated in SDS-PAGE without previously boiling the samples followed by immunoblotting (Fig. 4.3). One or two hours incubation of the F4 fimbriae at a low pH did not lead to a considerable degradation: after 1 hour at pH 1.5 only a very small part of the multimeric F4 fimbriae was converted into monomers, after 2 hours the monomeric forms were detected at pH 1.5 and 2 and after 2.5 hours also at pH 3. However, after 2.5 hours at pH 1.5 and 3.5 hours at pH 1.5 and 2, only di-and monomers of the major subunit were found whereas after 3.5 hours at pH 3 the major part of the F4 fimbriae were still in multimers.

The lower staining intensity of the monomers in Western blot after 3.5 hours at pH 1.5 and 2 as compared to pH 3 did not correspond with a weaker band intensity on the Coomassie stained gel indicating a loss of the three-dimensional structure of the monomer at pH 1.5 and 2.

A.



B.

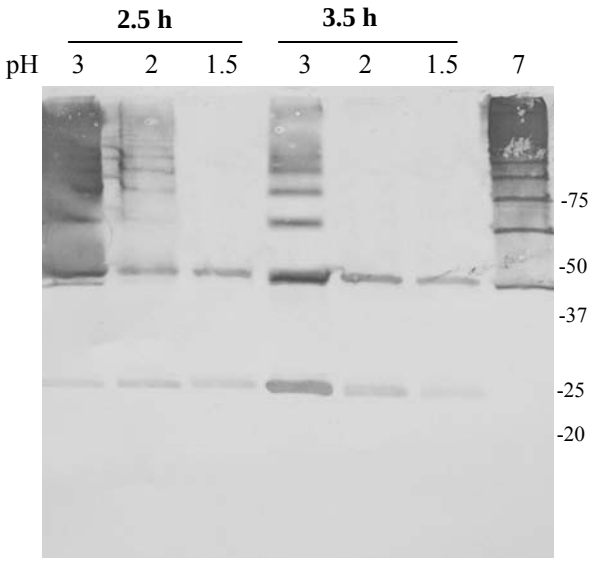
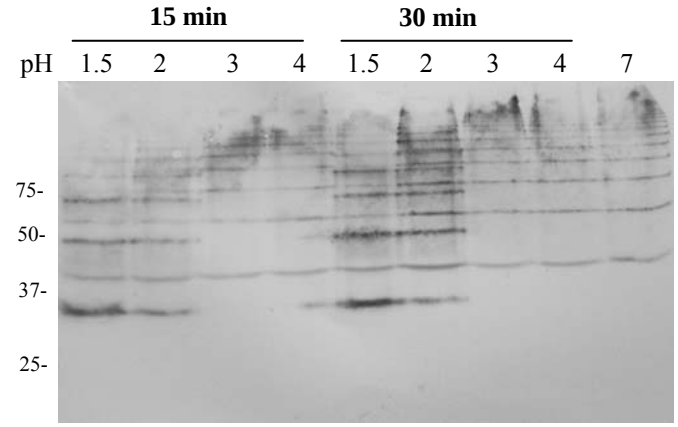


Fig. 4.3. Evaluation by immunoblotting of the stability of F4 fimbriae to pH 1.5, 2 and 3 during 1 and 2 hours (A) and during 2.5 and 3.5 hours (B); pH 7 is used as control sample. Molecular weight is indicated.

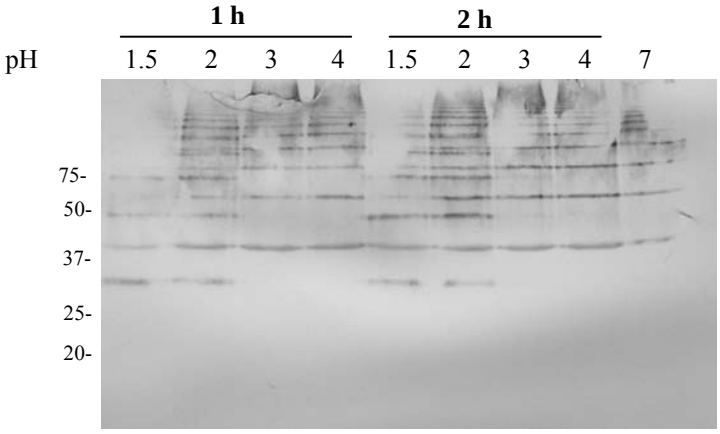
4.4.5. Effect of gastric digestion on the antigenicity of the F4 fimbriae

The effect of gastric digestion on F4 fimbriae was evaluated using SGF (United States Pharmacopeia, 1995) (Fig. 4.4). Since the SGF incubations were performed on different days, the proteolytic activity of each batch of SGF was estimated using a standard assay (Ryle, 1984). The proteolytic activity of SGF was 21,181 ± 4,142 units at pH 3. Since pepsin has no proteolytic activity at pH 7, incubation at this pH was used as control. Although degradation products of the F4 fimbriae were already observed after 15 and 30 min incubation at pH 1.5 and 2, respectively, the F4 fimbriae were still not completely digested after 2 hours incubation. However, after 3 and 4 hours, almost complete digestion was observed. At pH 3 and higher, the fimbriae were stable to gastric digestion for at least 4 hours.

A.



B.



C.

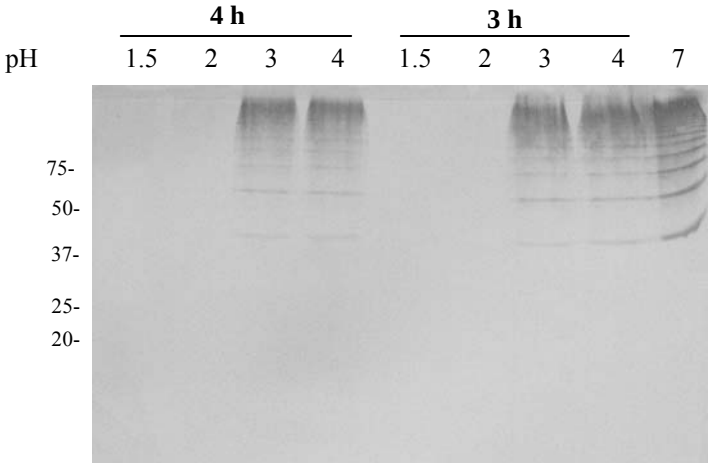


Fig. 4.4. Evaluation by immunoblotting of the stability of F4 fimbriae to gastric digestion in SGF at pH 1.5, 2, 3 or 4 during 15 en 30 min (A), 1 and 2 hours (B), 3 and 4 hours (C). Molecular weight is indicated.

4.5. DISCUSSION

Resistance of F4 fimbriae to gastric digestion is a prerequisite for its antigenicity. Knowledge about the stability to gastric digestion is lacking but is of crucial importance in the efficient design of an oral vaccine for piglets. Since no reports on the gastrointestinal pH in young piglets were available, we analysed the pH throughout the gastrointestinal tract of piglets at different time points around weaning and assessed *in vitro* the stability of the F4 fimbriae to gastric pepsin at different pH.

Compartments of distinct pH and specific enzymatic conditions are discerned in the pig's stomach (Liebler-Tenorio *et al.*, 1999). Saliva and cardiac glandular secretions maintain the pH in the cardiac gland region higher and permit continued activity of salivary α-amylase and bacterial fermentation of carbohydrates. In the fundic and pyloric glandular region, acid and pepsinogen are secreted. Pepsin, the hydrolytic product of pepsinogen, has proteolytic activity at low pH, and can consequently perform its activity in this region. In the present study, the pH was measured in different parts of the stomach of suckling piglets. The lowest gastric pH values were found in the fundus gland region (3.0 ± 0.3), while the highest values were recorded in the diverticulum (3.9 ± 0.9) and near the torus pyloricus (4.1 ± 0.1). The high pH near the torus pyloricus can be explained by reflux of duodenal secretions i.e. alkaline pancreatic juice and bile.

Although no significant differences in gastric pH were found between piglets of different ages, pH values below 2.5 were not observed in the suckling piglets at the day of weaning. This is in contrast with piglets 1 and 2 weeks postweaning, in which pH values are found as low as 1.6-1.7. The relatively high gastric pH in the suckling and the recently weaned piglet can be explained by several factors. Firstly, the stomach of these young piglets has a less developed capacity to secrete hydrochloric acid (Cranwell *et al.*, 1976). Secondly, the sow's milk is not strongly stimulating for this secretion, and has a considerably buffering capacity (Kvasnitskii, 1951). Thirdly, lactic acid production from lactobacilli in the upper alimentary tract of these piglets can partially suppress hydrochloric acid production (Cranwell *et al.*, 1976). The low gastric pH (1.6-1.7) in piglets 1 and 2 weeks postweaning, indicate that the gastric secretion is more developed

in these piglets, resembling that of the mature pig (Clemens *et al.*, 1975). The large variability in gastric pH in the weaned piglets could be due to different time-intervals between the last meal and the measurement, as the piglets received food ad libitum. Indeed for mature piglets, pH values as low as 1 to 2 are reported before a meal, while 4 to 5 immediately after the meal depending on the diet and the feeding regime (Lawrence, 1970, 1972; Argenzio and Southworth, 1974; Clemens *et al.*, 1975; Potkins *et al.*, 1991). Subsequently, the pH falls over the next 4 to 8 h, the rate of decline depending both on the composition and the physical nature of the diet (pellets, meal, crumbles) (Lawrence, 1970, 1972).

Much smaller variations in pH were observed at any point along the mid and caudal small and large intestine than in the stomach and 1 cm distal from the pylorus, indicating that the digesta, which enter this part of the intestine are already effectively buffered by pancreas secretions and bile. This observation is supported by Clemens *et al.* (1975), and Braude *et al.* (1976) who studied the GI pH in the mature pig.

In all age groups, the pH increased significantly with increasing distance from the stomach, to reach maximum values at 3/4 and 4/4 of the small intestine. In the caecum, the pH decreased again to values seen in the proximal jejunum (1/16). A similar change in pH along the gut was also seen in adult pigs (Keys and DeBarthe, 1974; Clemens *et al.*, 1975; Potkins *et al.*, 1991). In the first half of the small intestine and the caecum, the pH decreased with age, while no significant influence of age was seen in caudal half of the small intestine, the colon and rectum.

The present study demonstrates that acidity alone had no major effect on the antigenicity of the F4 fimbriae. Only after relatively long incubation periods (2.5 to 3.5 hours) at pH 1.5 and 2, the F4 multimers were converted into di-and monomers. Incubation in GSF at pH 1.5 and 2 during the first 2 hours resulted only in a limited degradation of the fimbriae whereas from 3 hours onwards a complete digestion and thus loss of antigenicity occurred. At pH 3 the F4 fimbriae maintained their antigenicity, even though the SGF has high proteolytic activity as measured on haemoglobin (Ryle, 1984). Indeed, different pH optima for protease activity are reported depending on the substrate

(Maki and Yanagisawa, 1986; Young *et al.*, 1995) and could explain the discrepancy for digestion at pH 3 between haemoglobin and the fimbriae.

Liquids, which empty much faster than solids (Camilleri *et al.*, 1985; Gregory *et al.*,1990), pass the pig's stomach relatively quickly: in suckling piglets, the stomach is almost completely emptied within 2 hours after drinking (Kidder *et al.*, 1961; Kidder and Manners, 1968), whereas in growing and adult pigs, only 50 % has entered the small intestine after 1.5 to 2 hours and 70 and 80 % after 4 hours (Clemens *et al.*, 1975; Gregory *et al.*, 1990; Davis *et al.*, 2001). However, the stress of weaning prolongs the transit times leading to long gastric residence times shortly after weaning (Snoeck *et al.*, 2004a). The present results suggest that from one week postweaning onwards, only a minor part of the F4 fimbriae will lose its antigenicity due to gastric digestion since 50 % of them will have passed the stomach within 2 hours. However if the F4 fimbriae are orally administered shortly postweaning, a major part of the F4 fimbriae can be digested in the stomach, considering the long gastric residence time shortly postweaning (Snoeck *et al.*, 2004a). The higher gastric pH values in combination with the faster gastric emptying in the suckling piglets, suggest that also in this group the major portion of the F4 fimbriae will maintain its antigenicity upon oral immunisation.

4.6. CONCLUSION

The results of the present study indicate that gastric digestion will only have a limited impact on oral immunisation with F4 fimbriae, as long as the immunisation does not take place shortly postweaning. Shortly after weaning, it might be necessary to protect the F4 fimbriae against gastric digestion to obtain an efficient immunisation of the piglets. Protecting the F4 fimbriae with an enteric coating that dissolves in the caudal part of the small intestine could be favourable. In suckling piglets, milk itself should also be taken into account (Snoeck *et al.*, 2003) since F4-specific milk antibodies can inhibit the adherence of F4 fimbriae to their receptors and so their oral immunogenicity. In this case also, protecting the F4 fimbriae by enteric coating could be beneficial.

4.7. ACKNOWLEDGEMENTS

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CHAPTER 5

THE JEJUNAL PEYER'S PATCHES ARE THE MAJOR INDUCTIVE SITES OF THE F4-SPECIFIC IMMUNE RESPONSE FOLLOWING INTESTINAL IMMUNISATION OF PIGS WITH F4 FIMBRIAE

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5.1. ABSTRACT

Although newly weaned pigs can be orally immunised with F4 (K88) fimbriae against an F4$^+$ *Escherichia coli* infection, the inductive sites of the F4-specific intestinal immune response have to be defined to determine the most effective way of vaccination. Therefore, newly weaned pigs were immunised orally, in the lumen of the mid-jejunum, the ileum or the mid-colon with isolated F4 fimbriae to determine which of these immunisations generate the greatest level of antibodies in the small intestine. Following a single immunisation, F4-specific antibody secreting cells (ASC) in the small intestine were enumerated by ELIspot, and F4-specific antibody titers in serum and saponin extracts of the jejunum were determined by ELISA. Each immunisation similarly increased F4-specific IgM and IgA in serum, except for the colonic immunisation which failed to evoke F4-specific serum antibodies. On the contrary, F4-specific serum IgG titers were significantly higher following oral immunisation. Throughout the small intestine, the highest number of F4-specific ASC was found following jejunal immunisation, followed by ileal and oral immunisation. After colonic immunisation, the F4-specific ASC in the small intestine were restricted to the ileum. Determination of the F4-specific antibodies in saponin extracts of the jejunum demonstrated diffusion of serum antibodies in the intestinal lamina propria (LP). To elucidate the importance of the jejunal Peyer's patches (JPP), the jejunal LP and the ileal Peyer's patches (IPP) in the induction of the immune response, the F4 fimbriae were selectively targeted to the JPP, jejunal LP or IPP by local injection in the intestinal wall. Similar but low F4-specific serum antibodies were evoked following each immunisation. However, the highest number F4-specific ASC in the small intestine was found following immunisation in the JPP, whereas immunisation in the jejunal LP and IPP resulted in a lower and similar intestinal antibody response. In summary, the results show that the JPP are the major inductive sites of the F4-specific intestinal antibody response. On the contrary, the IPP seems to be of less importance as induction site. Consequently, selective targeting of the F4 fimbriae to the JPP will allow the most effective way of vaccination.

5.2. INTRODUCTION

F4+ enterotoxigenic *Escherichia coli* (ETEC) are an important cause of diarrhoea and mortality in piglets immediately after weaning causing significant economic losses (Hampson, 1994). At this moment, there are no commercial vaccines against this F4+ETEC-induced postweaning diarrhoea. Nevertheless, newly weaned piglets can be orally immunised against F4+*E.coli* infection with purified F4 fimbriae (Van den Broeck *et al.*, 1999a). Although the isolation and identification of antigens are important steps in vaccine development, it is also crucial to examine the inductive sites of the immune response to determine the most effective way of vaccination. Presence of receptors for the F4 fimbriae on the small intestinal enterocytes is a prerequisite for inducing the protective mucosal immune response following oral immunisation (Van den Broeck *et al.*, 1999a). Since the F4 fimbriae can adhere to the F4-specific receptors present on villous enterocytes throughout the small intestine (Cox and Houvenaghel, 1993), the question rose whether binding of the F4 fimbriae by the villous enterocytes in the cranial part of the small intestine plays a role in induction of the immune response and whether binding to enterocytes and M cells in the dome epithelium of the Peyer's patches, present in the more caudal parts of the jejunum and in the ileum, would result in a better immune response. Therefore, the F4-specific antibody response following local injection of the F4 fimbriae in the lumen of the mid-jejunum or the ileum was compared to oral immunisation. Furthermore, immunisation in the colon was performed to investigate whether F4 fimbriae reaching the colon still can contribute to the F4-specific immune response in the small intestine. To determine the importance of the Peyer's patches and lamina propria in the induction of the immune response, the F4 fimbriae were selectively targeted to the lamina propria (LP), the jejunal (JPP) or ileal Peyer's patches (IPP) by local injection, after which the F4-specific antibody response was studied.

5.3. MATERIAL AND METHODS

5.3.1. Experimental animals

Twenty-seven piglets (Belgian Landrace x Piétrain or Belgian Landrace x Dutch Landrace, 4 litters), seronegative for antibodies against F4 as determined by ELISA, were

weaned at the age of 3.5 to 4 weeks and subsequently housed in isolation units at 24 ± 2 °C with IR-lamps for local heating with food and water ad libitum. Starting one day before weaning, all animals were orally treated during 3 successive days with colistine (150,000 U/kg body weight; Colivet, Prodivet Pharmaceuticals, Eynatten, Belgium) to prevent outbreaks of *E. coli* infections during the weaning period. Experimental procedures and animal management procedures were undertaken in accordance with the requirements of the animal care and ethics committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2003/58).

5.3.2. Purification of F4 fimbriae

The F4 fimbriae were purified as described by Van den Broeck *et al.* (1999c). Briefly, fimbriae were isolated by homogenizing the bacterial suspension of *E. coli* strain GIS 26 (O149:K91:F4ac, LT^+, STa^+, STb^+) using an Ultra Turrax (Janke & Kunkel, IKA Labortechnik, Staufen, Germany). The solubilised fimbriae were subsequently precipitated with 40% (w/v) ammonium sulphate, after which the pellet was dissolved and dialysed overnight against PBS. The protein concentration of the isolated solution was determined using the bicinchoninic acid protein assay kit (Sigma-Aldrich, Bornem, Belgium). The purity was assessed by electrophoresis on a SDS-12% polyacrylamide slab gel, followed by analysis of the Coomassie stained gel using the gel analysis software, Image Master 1D® (Amersham Pharmasia biotech, Newcastle upon Tyne, England), so that the concentration of the F4 fimbriae in the solution could be determined.

5.3.3. Experimental procedure

Experiment 1.

In the first experiment, 17 pigs were used. Seven days postweaning, 4 animals were orally given 4 mg F4 fimbriae in PBS (oral group) as further described. At the same time, groups of 4 animals were locally injected with F4 fimbriae (4 mg in PBS) in the lumen of the mid-jejunum, the ileum or the mid-colon (jejunum, ileum and colon group, respectively) following laparotomy as further described. In the jejunum group, one pig was injected in the jejunal lumen in between 2 discrete PP, which were located 3 cm from each other, whereas in the other pigs of this group no PP were present nearby the

injection site. One animal was a non-immunised control. The F4-specific serum IgM, IgA and IgG response was determined 0, 7, 14 and 21 days post immunisation (dpi). The intestinal antibody response was quantified and localised by enumerating the F4-specific antibody-secreting cells (ASC) in the lamina propria of the proximal jejunum and the mid-jejunum, in the jejunal and ileal Peyer's patches, and in the mesenteric lymph nodes draining the mid-jejunum for 2 piglets of each group at 14 dpi and for the remaining 2 piglets at 21 dpi. The non-immunised control piglet was analysed at 7 dpi. Tissues were taken following euthanasia of animals by intravenous injection of an overdose of pentobarbital (24 mg/kg; Nembutal, Sanofi Santé Animale, Brussels, Belgium) and subsequent exsanguination. Furthermore, small intestinal segments (2 cm^2) were sampled from the proximal jejunum and from the mid-jejunum for measuring the F4-specific IgM, IgA and IgG antibodies in tissue extract. Finally, jejunal villi were isolated for determining the presence of F4 receptors (F4R) on the villous enterocytes by an *in vitro* villous adhesion assay to confirm the susceptibility of the piglets to F4$^+$*E.coli* infection.

Experiment 2.

In the second experiment 10 pigs were used. Seven days postweaning, 4 mg F4 fimbriae was locally injected in the jejunal lamina propria (3 animals, LP group), the jejunal Peyer's patches (2 animals, JPP group) or the ileal Peyer's patches (3 animals, IPP group) following laparotomy. Two animals were non-immunised controls. The F4-specific serum IgM, IgA and IgG response was determined 0, 7 and 14 dpi, and additionally at 21 dpi for the non-immunised controls. Except for the tissue extracts and the mesenteric lymph nodes, the same samples were taken following euthanasia as in the first experiment at 14 dpi for the immunised and at 21 dpi for the non-immunised control piglets.

5.3.4. Oral immunisation and local injection of F4 fimbriae in the intestinal lumen or wall

The oral group was deprived of food and water from 3 h before, until 2 h after the oral administration of the F4 fimbriae (4 mg F4 fimbriae in 5 ml PBS with a syringe) to allow an optimal uptake of the antigen and to enhance the passage through the stomach.

For local immunisation in the gut wall or lumen, the piglets were fasted overnight, after which they were anesthetised with tiletamine and zolazepam (Zoletil 100; Virbac,

Louvain La Neuve, Belgium) supplemented with 2% Xylazine (V.M.D., Arendonk, Belgium) (0.22 ml/kg body weight, intramuscular, IM). Laparotomy was performed in the left flank and the F4 fimbrial solution (4 mg F4 in 5 ml PBS) was injected with a syringe via a 26 G needle. When the injection occurred in the intestinal lumen, the needle was flushed with sterile water before it was withdrawn from the lumen to avoid leakage of the F4 solution into the wall. When the injection occurred in the intestinal wall, 5 sites over a length of 15 cm (jejunal LP and IPP) or 5 sites at several (3 to 4) jejunal PP were injected with 1 ml F4 solution per site. After the surgery, the piglets were deprived of food for an additional 24 h but they obtained water ad libitum. Furthermore, they were treated with amoxicilline (10 mg/kg body weight, IM, Codimox Long Acting, Codifar, Wommelgem, Belgium) for 1 week and flunixine (1.8-3.7 mg/kg body weight, IM, Finadyne, Schering-Plough, Brussels, Belgium) during 3 days.

5.3.5 Samples

5.3.5.1. Serum

Blood was sampled from the jugular vein. After 18 h incubation at room temperature (RT), serum was collected and inactivated at 56 °C during 30 min and subsequently treated with kaolin (Sigma-Aldrich, Bornem, Belgium) to decrease the background reading in the ELISA (Van den Broeck *et al.*, 1999a). Before testing, the serum was diluted in ELISA dilution buffer (PBS + 0.05% (v/v) Tween®20 (Merck, Hohenbrunn, Germany) + 3% (w/v) bovine serum albumin (BSA; Sigma-Aldrich, Bornem, Belgium) to obtain a final serum dilution of 1/10.

5.3.5.2. Lamina propria monomorphonuclear cells (MC)

Immediately after euthanasia, an intestinal segment without Peyer's patches was excised from the first 2 meters of the jejunum (LP_{prox}) and the mid-jejunum (LP_{mid}) and flushed with PBS containing penicillin (100 IU/ml, GIBCO BRL, Merelbeke, Belgium) and streptomycin (100 µg/ml, GIBCO BRL) at RT to remove the intestinal content. Subsequently, the segment was cut into pieces of 4 cm^2 and a total amount of 60 g was used for the isolation of MC. The MC were further isolated as described by Verdonck *et*

al. (2002). The number of F4-specific IgM, IgA or IgG ASC per 10^7 MC was enumerated by ELIspot and the number F4-specific IgM, IgA or IgG ASC/g LP was calculated for evaluation of correlation with F4-specific IgM, IgA or IgG titers in jejunal tissue extracts and serum.

5.3.5.3. Peyer's patches and mesenteric lymph node MC

Immediately after euthanasia, jejunal and ileal Peyer's patches were excised from the intestine and mesenteric lymph nodes draining the mid-jejunum were dissected. The MC were isolated as described by Verdonck *et al.* (2002).

5.3.5.4. Extracts of proximal part of the jejunum and mid-jejunum

At the moment of euthanasia, two small intestinal segments (2 cm^2) without Peyer's patches and 5 cm apart were excised from the proximal jejunum (J_{prox}) and the same was done in the mid-jejunum (J_{mid}). Subsequently, the segments were washed with PBS at RT and cut into pieces of about 3 mm^3 which were immediately frozen at -70°C. Antibodies were extracted from these tissues as described by Bergquist *et al.* (2000). Briefly, 40 mg tissue was thawed in 300 µl PBS with 2% (w/v) saponin, 0.35 mg/ml phenylmethylsulfonyl fluoride (PMSF, Roche Diagnostics, Mannheim, Germany) and 0.1% (w/v) BSA (Sigma-Aldrich) overnight at 4°C. PMSF was added to prevent enzymatic degradation of the samples. The samples were inactivated at 56°C during 30 min and subsequently centrifuged at 13,800 x *g* for 5 min and the supernatant was stored at – 70°C until analysis in ELISA.

5.3.6. ELISA for F4-specific IgG, IgA and IgM

F4-specific antibodies in serum and tissue extracts were determined using the indirect ELISA described by Van der Stede *et al.* (2003). The optical density was measured at 405nm (OD_{405}) after 30 min incubation. The serum IgM, IgA and IgG cutoff values were calculated as the mean OD_{405} of all sera (dilution 1/10) at 0 dpi, increased with three times the standard deviation (SD). The IgM, IgA and IgG cutoff values for antibodies in tissue extracts were calculated as the mean OD_{405} of the J_{prox} and J_{mid} extracts (dilution 1/8.5) of the control animal increased with three times the SD. The antibody titer was the inverse

of the highest dilution which still had an OD_{405} higher than the calculated cutoff value. The obtained cutoff values were 0.33, 0.38, 0.46 and 0.35, 0.27, 0.50 for F4-specific serum IgM, IgA and IgG of the first and second experiment, respectively; 0.15, 0.37 and 0.39 for F4-specific IgM, IgA and IgG of the tissue extracts, respectively. The antibody titer in J_{prox} extracts was determined separately in both segments and subsequently averaged to obtain the final antibody titer. The same was done for both J_{mid} extracts.

5.3.7. ELIspot assay for F4-specific IgM, IgA and IgG antibody secreting cells (ASC)

The ELIspot assay was performed as described by Verdonck *et al.* (2002) with following modifications: the microtiter plates were coated with the F4-specific monoclonal antibody [IMM01 (Van der Stede *et al.*, 2002), 1µg/ml in PBS (150mM, pH=7.4)]. Subsequently, the plates were incubated with purified F4 (25 µg/ml in ELISA dilitution buffer), MC suspensions at a concentration of 10^7 cells/ml leukocyte medium (100 µl per well), optimal dilutions of biotinylated-swine-specific IgM, IgA and IgG Mab and HRP-conjugated streptavidin. The substrate solution consisted of AEC, H_2O_2 and low-melting point agarose. For each MC suspension, spots in five wells (10^6 MC per well) were counted to obtain the number of isotype-specific ASC per 5 x 10^6 MC. Results are presented as the number of ASC per 10^7 MC.

5.3.8. *In vitro* villous adhesion assay for F4R characterisation of the piglets

In order to determine the presence of F4R on the small intestinal villous enterocytes, an *in vitro* villous-adhesion assay was performed as described by Van den Broeck *et al.* (1999c). Adhesion of more than 5 bacteria per 250 µm villous brush border length was noted as positive (Cox and Houvenaghel, 1993).

5.3.9. Statistics

The data were tested for normal distribution with a Kolmogorov-Smirnov test and the homogeneity of variances was tested with a Levene's test. Statistical analysis was performed using the software package SPSS version 11.0. Differences in $\log_2$ antibody titers (IgM, IgA, IgG) between the groups and between different time points within the groups were tested for statistical significance using General Linear Model (Repeated

Measurements) using the following syntax: GLM; Time1 time 2 time 3….time x BY group; /WSFACTOR = time x SIMPLE; /METHOD = SSTYPE (3); /CRITERIA = ALPHA (.05); /EMMEANS = TABLES (group*time) COMPARE (group); /EMMEANS = TABLES (group*time) COMPARE (time); /WSDESIGN = time; /DESIGN = group

The relationship between the $\log_2$ antibody titers (IgM, IgA, IgG) in jejunal tissue extracts and ASC/g tissue (IgM, IgA, IgG) in LP or $\log_2$ serum antibody titers (IgM, IgA, IgG) was estimated by the Spearman's correlation test. Statistical significance was defined at $P<0.05$, unless stated otherwise.

5.4. RESULTS

5.4.1. Experiment 1

5.4.1.1. F4R characterisation of the piglets

The *in vitro* villous adhesion assay showed that all animals possessed the F4R at their villous brush border. The number of F4$^+$*E. coli* adhering per 250 µm villous brush border length ranged from 20.5 to 114 with a median of 53.

5.4.1.2. F4-specific serum antibody responses following immunisation

The F4-specific serum antibody response was determined 0, 7, 14 and 21 dpi. The F4-specific serum antibody response was independent of the degree of F4R expression. No F4-specific serum antibodies were detected in the control animal and in the pigs of the colon group. In the other groups, the F4-specific IgM and IgA serum antibody response was highest 7 and 14 dpi (Fig. 5.1). The IgM titers in the oral ($P<0.05$, 7 dpi), jejunum ($P<0.1$, 14 dpi) and ileum ($P<0.1$, 7 dpi) group increased significantly post immunisation, but were not significantly higher than in the colon group. Furthermore, F4-specific serum IgA titers were detected in the oral (7 dpi), the jejunum (14 dpi), and the ileum group (7 dpi). In contrast, the F4-specific IgG titers only clearly increased in the oral group and became significantly higher than in all other groups ($P<0.05$), where they remained near baseline levels.

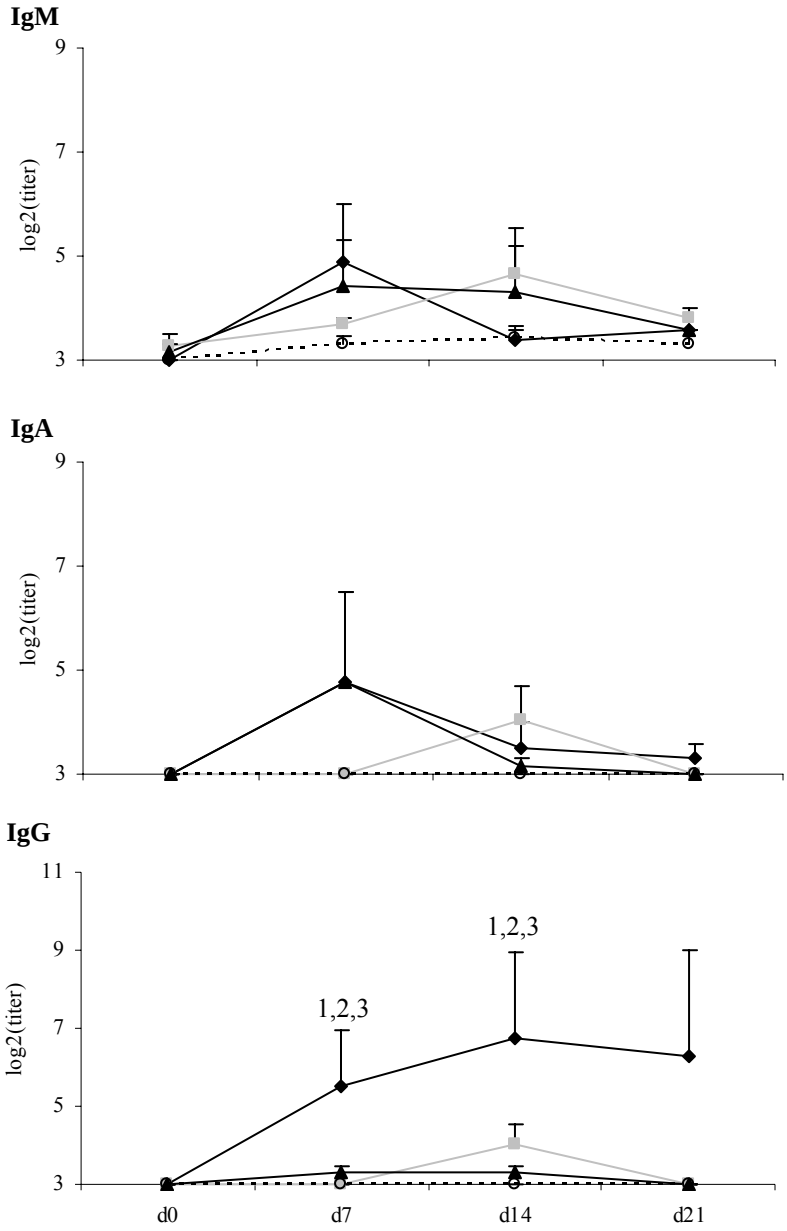

Fig. 5.1. Mean F4-specific IgM, IgA and IgG serum antibody titers (± SEM) at 0, 7, 14 and 21 following oral immunisation (◆), or local injection of the F4 fimbriae in the lumen of the jejunum (■), the ileum (▲) and the colon (○). At 0, 7 and 14 days post-immunisation (dpi) n=4, at 21 dpi n=2. In the control animal, euthanised at 7dpi, no F4-specific serum antibodies were detected. Significant differences between the oral group and the jejunum, ileum and colon group were indicated with ^{1,2,3}, respectively.

5.4.1.3. F4-specific antibody secreting cells in the intestine following immunisation

In order to localise the induced antibody response, the number of F4-specific IgM, IgA and IgG ASC was determined in lamina propria of the proximal jejunum (LP_{prox}) and the mid-jejunum (LP_{mid}), in the jejunal (JPP) and ileal Peyer's patches (IPP), and in the mesenteric lymph nodes draining the mid-jejunum (MLN) for 2 piglets of each immunised group at 14 dpi and for the remaining 2 piglets at 21 dpi. The number of F4-specific APC was independent on the degree of expression of the F4R at the villous brush borders. In the non-immunised control pig, euthanised at 7 dpi, a background of F4-specific IgM ASC was detected in the LP_{prox} (3 IgM ASC per 10^7 MC), but no F4-specific IgG or IgA ASC were identified.

F4-specific IgM ASC were detected in all immunised groups (Fig. 5.2.). The high response in the jejunum group at 14dpi resulted mainly from the pig immunised in the jejunal lumen in between 2 discrete PP, whereas in the other pigs of this group no PP were present nearby the injection site. In the LP_{prox}, the highest number IgM ASC were found in the jejunum (up to 20 IgM ASC per 10^7 MC) and oral groups (up to 10 IgM ASC per 10^7 MC), whereas in the ileum and colon groups the response was low to absent. In the LP_{mid}, no major differences between the different groups were found. Slightly higher IgM ASC numbers were identified in the jejunum and ileum groups (up to 10 and 6 IgM ASC per 10^7 MC, respectively). In the JPP, the highest numbers IgM ASC were found in the jejunum group (up to 62 IgM ASC per 10^7 MC) followed by the ileum and the oral groups (up to 30 IgM ASC per 10^7 MC), whereas the response was low in the colon group. In the IPP, high numbers of IgM ASC were found in the jejunum (up to 83 IgM ASC per 10^7 MC), the ileum, colon and oral groups (up to 40, 34 and 28 IgM ASC per 10^7 MC, respectively). In the MLN draining the mid-jejunum, the highest response was found in the jejunum group (up to 65 IgM ASC per 10^7 MC), whereas much lower numbers were found in the other groups.

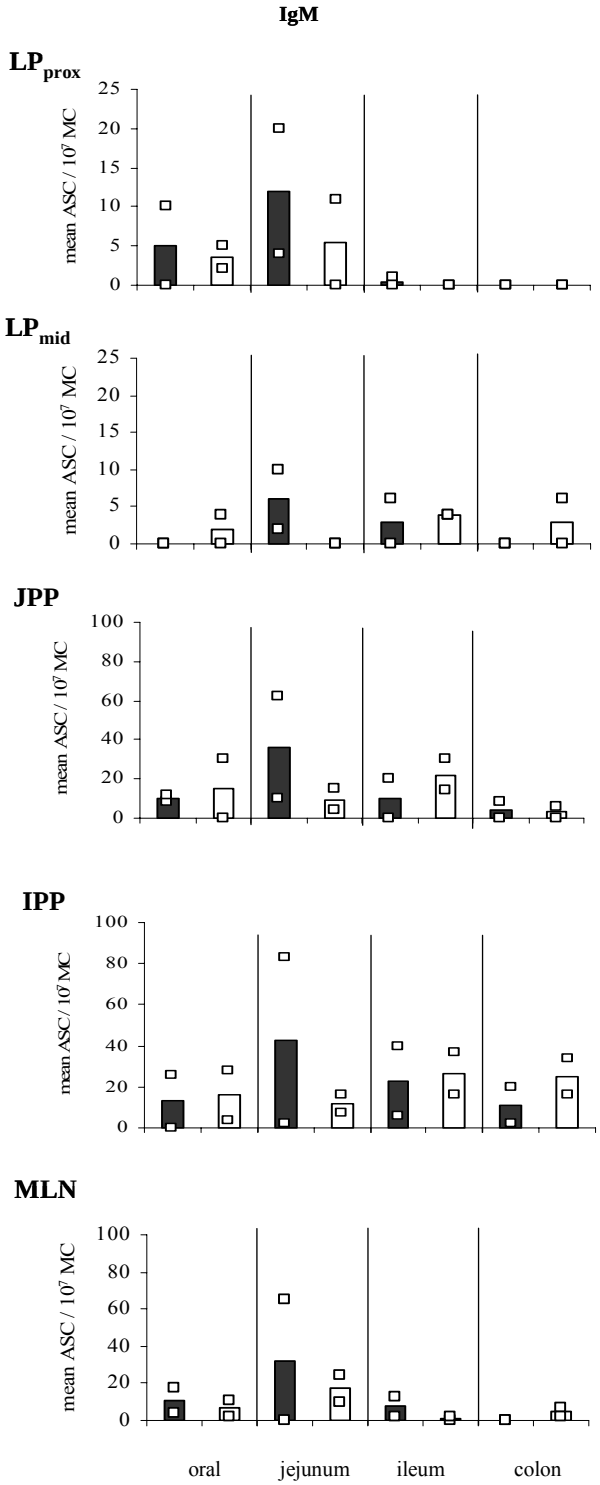

Fig. 5.2. F4-specific IgM ASC per 10^7 MC in lamina propria of the proximal jejunum (LP_{prox}) and the mid-jejunum (LP_{mid}), jejunal (JPP) and ileal Peyer's patches (IPP), and mesenteric lymph nodes draining the mid-jejunum (MLN) following oral immunisation, or local injection of the F4 fimbriae in the lumen of the jejunum, the ileum or the colon at 14 (black bars) and 21 (white bars) days post-immunisation (dpi). At 14 dpi and at 21 dpi n= 2, except for the LP_{mid} in the ileum group at 21 dpi n =1. The vertical bars represent means, and the open squares indicated values for individual pigs.

The highest F4-specific IgA ASC were detected in the ileum group (Fig 5.3). In this group up 12 and 20 IgA ASC per 10^7 MC were detected in the LP_{mid} at 21 dpi and in the JPP at 14 dpi, respectively. Lower numbers to no ASC were found in the IPP and MLN, whereas none could be identified in the LP_{prox}. Only few IgA ASC were detected in all tissues in the oral group, in the LP_{mid}, JPP and MLN in the jejunum group and in the MLN in the colon group, at 14 or 21 dpi.

F4-specific IgG ASC could only be found in the JPP, IPP and MLN of the oral and the jejunum group and this mainly at 21 dpi, except for a few IgG ASC in the IPP of the colon group (Fig. 5.3).

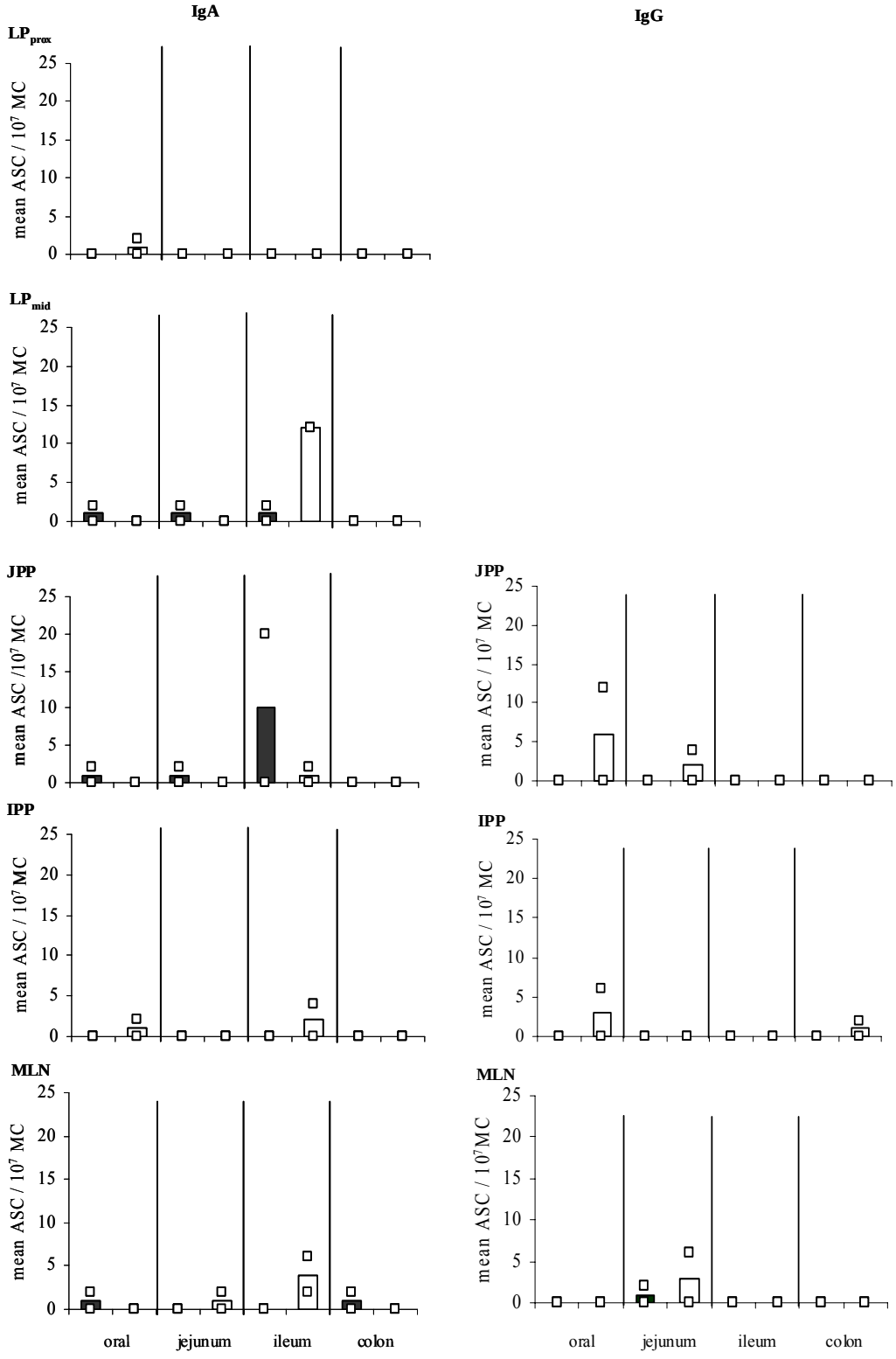

Fig. 5.3. F4-specific IgA and IgG ASC per 10^7 MC in lamina propria of the proximal jejunum (LP_{prox}) and the mid-jejunum (LP_{mid}), jejunal (JPP) and ileal Peyer's patches (IPP), and mesenteric lymph nodes draining the mid-jejunum (MLN) following oral immunisation, or local injection of the F4 fimbriae in the lumen of the jejunum, the ileum or the colon at 14 (black bars) and 21 (white bars) days post-immunisation (dpi). At 14 dpi and at 21 dpi n=2, except for the LP_{mid} in the ileum group at 21 dpi, n =1. No IgG ASC could be found in both the LP_{prox} as the LP_{mid}. The vertical bars represent means, and open squares indicate values for individual pigs.

5.4.1.4. F4-specific antibody response in the jejunum following immunisation

In addition to the F4-specific ASC, the induced intestinal antibody response was analysed by determining the F4-specific IgM, IgA and IgG antibody titers in extracts of the proximal jejunal (J_{prox}) and the mid-jejunal tissues (J_{mid}) (Fig. 5.4).

F4-specific IgM antibodies were found in all groups both in the proximal an the mid-jejunum, with the lowest titers detected in the colon group. F4-specific IgA antibodies were found in the oral, the jejunum and the ileum groups, but only in the oral and the ileum groups, they were detected in both the J_{prox} as the J_{mid}, whereas in the jejunum group they were only found in the J_{mid}. F4-specific IgG antibodies were only found in the J_{prox} of the oral group 21 dpi (data not shown).

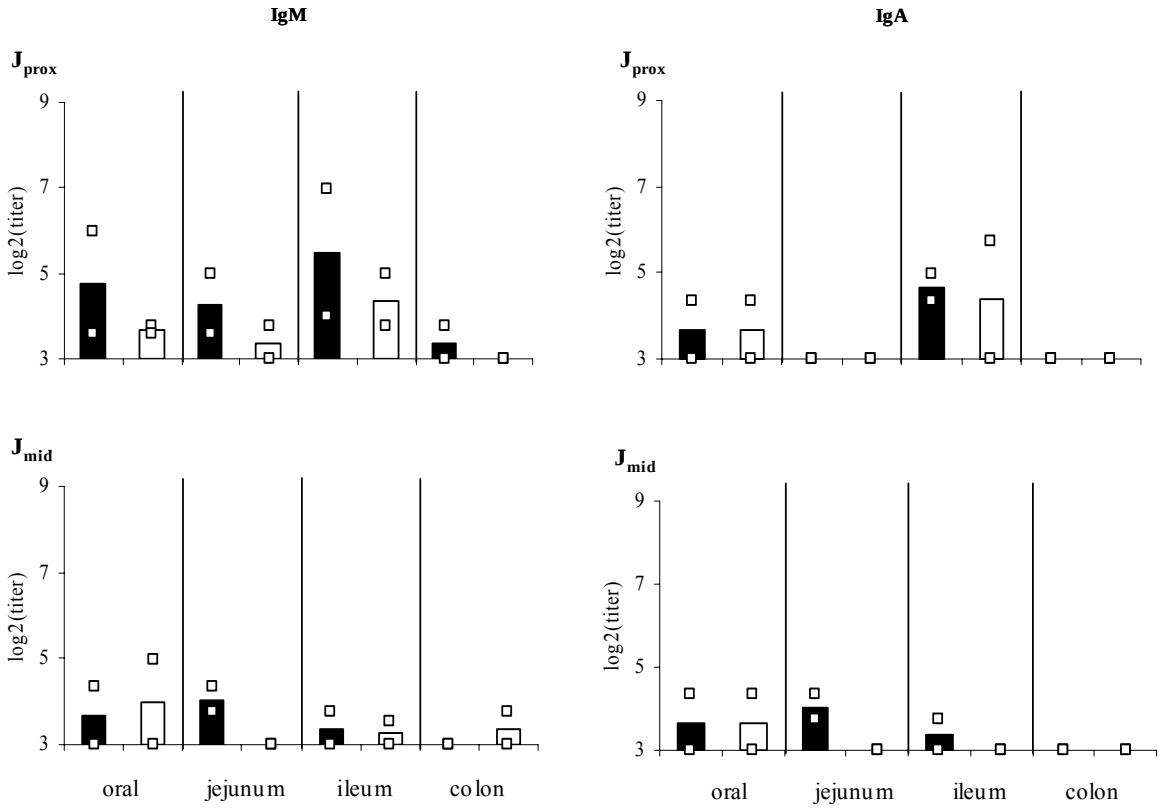

Fig.5.4. F4-specific IgM and IgA antibody titers [log_2 (titer)] in tissue extracts of the proximal jejunum (J_{prox}) and the mid-jejunum (J_{mid}) following oral immunisation, or local injection of the F4 fimbriae in the lumen of the jejunum, the ileum or the colon at 14 (black bars) and 21 (white bars) days post-immunisation (dpi). At 14 dpi and 21 dpi n=2. The vertical bars represent means, and open squares indicate values for individual pigs.

5.4.1.5. Correlation between the amount of F4-specific ASC in the LP and F4-specific antibodies in jejunal tissue or serum

The IgM, IgA and IgG antibody titers in J_{prox} and J_{mid} extracts was often, but not always corresponding with the number of IgM, IgA and IgG ASC/g tissue in LP_{prox} and LP_{mid}. Therefore, the antibody titer in tissue extracts and the number of ASC/g tissue correlated only poorly. A better correlation was seen if the jejunum was taken as a whole (no subdivision into the proximal and mid-jejunum). For this purpose, the antibody titer in the J_{prox} and the J_{mid} extracts, as well as the number of ASC/g LP_{prox} and LP_{mid} were averaged for each animal. The correlation coefficient for IgM and IgA were 0.472 ($P<0.05$) and 0.827 ($P< 0.01$), respectively. F4-specific IgG antibodies were only found in the J_{prox} at 21 dpi in the oral group, whereas no IgG ASC were found in the LP in this group.
Furthermore, both the titers in jejunal tissue extracts as well as the number of ASC/g LP were not positively correlated with the serum IgM but weakly with the serum IgA titer on the day of euthanasia. The correlation coefficients between the serum IgM titer and the IgM titer in jejunal tissue extracts or the number of IgM ASC/g LP were -0.300 and -0.123, respectively. Those between the serum IgA titer and the IgA titer in jejunal tissue extracts or the number of IgA ASC/g LP were 0.499 ($P<0.05$) and 0.655 ($P<0.01$), respectively. On the contrary, the IgG antibody titer in jejunal tissue extracts was stongly correlated with the serum IgG titer ($r = 0.987$, $P<0.01$), whereas no IgG ASC in the LP were found.

5.4.2. Experiment 2

5.4.2.1. F4R characterisation of the piglets

The *in vitro* adhesion assay demonstrated that 5 of the 10 pigs lacked the F4-receptor on their villous enterocytes (1 of the LP, 1 of the JPP and 2 of the IPP group, respectively). In the remaining pigs, the number of $F4^+$*E. coli* adhering per 250 µm villous brush border length ranged from 41 to 74 with a median of 51.

5.4.2.2. F4-specific serum antibody response following immunisation

No F4-specific serum antibody response was identified in both non-immunised control animals. The F4-specific serum antibody response in the immunised pigs was

overall very low and no difference could be noticed between the differently immunised animals (data not shown). All immunised pigs developed an F4-specific IgM response, with titers ranging from 15 to 40. Similarly, F4-specific IgG responses, with titers ranging from 15 to 40, were identified in all animals except for one pig. On the contrary, F4-specific IgA responses, with titers ranging from 15 to 20, could only be identified in one pig out of each group and was irrespective of the F4R status of the pigs.

5.4.2.3. F4-specific antibody secreting cells in the intestine following immunisation

The antibody response induced in the intestine was studied by enumerating the number of IgM, IgA and IgG ASC in the lamina propria of the proximal jejunum (LP_{prox}) and the mid-jejunum (LP_{mid}), in the jejunal (JPP) and the ileal Peyer's patches (IPP) at 14 dpi for the immunised pigs (n=8), and at 21 dpi for the non-immunised control pigs (n=2). In both non-immunised controls no F4-specific ASC were identified.

Overall the number of F4-specific ASC was low and irrespective of the F4R status of the animals (Table 1). By far the highest numbers of F4-specific ASC were found following immunisation in the JPP (n=2). In this group, 20 to 30 IgM ASC per 10^7 MC were found in the IPP, whereas only 10 IgM ASC per 10^7 MC were detected in the JPP of one of both piglets (Table 1). Lower numbers of IgM ASC were found following immunisation in the LP (n=3) and IPP (n=3), namely up to 18 IgM ASC per 10^7 MC in the IPP and up to 4 IgM ASC per 10^7 MC in the JPP. In none of the animals, F4-specific IgM ASC were identified in the LP.

Only low numbers of F4-specific IgA ASC were identified following immunisation in the JPP, namely 2 to 4 IgA ASC per 10^7 MC in the JPP. Furthermore, 2 IgA ASC per 10^7 MC were found in the LP_{mid} of one pig immunised in the IPP. In none of the other pigs, F4-specific IgA ASC were detected.

F4-specific IgG ASC were detected following immunisation in the LP as well as in the JPP ranging from 2 to 4 IgG ASC per 10^7 MC.

Table 5.1.: The number of F4-specific ASC per 10^7 MC in the lamina propria of the proximal jejunum (LPprox) and the mid-jejunum (LPmid), the jejunal (JPP) and the ileal PP (IPP) on day 14 following immunisation in the intestinal wall at the jejunal LP, the JPP or the IPP. Furthermore, the number $F4^+$ *E.coli* adhering per 250 µm villous brush border length is given for all pigs.

Number of ASC per 10^7 MC	**Immunisation in the LP**			**Immunisation in the JPP**		**Immunisation in the IPP**		
IgM ASC per 10^7MC	Pig 1	Pig 2	Pig 3	Pig 4	Pig 5	Pig 6	Pig 7	Pig 8
LPprox	0	0	0	0	0	0	0	0
LPmid	0	0	0	0	0	0	0	**2**
IPP	**2**	**8**	0	**30**	**20**	**18**	**4**	0
JPP	**4**	0	0	0	**10**	0	**4**	0
IgA ASC per 10^7MC	Pig 1	Pig 2	Pig 3	Pig 4	Pig 5	Pig 6	Pig 7	Pig 8
LPprox	0	0	0	0	0	0	0	0
LPmid	0	0	0	0	0	0	0	**2**
IPP	0	0	0	0	0	0	0	0
JPP	0	0	0	**2**	**4**	0	0	0
IgG ASC per 10^7MC	Pig 1	Pig 2	Pig 3	Pig 4	Pig 5	Pig 6	Pig 7	Pig 8
LPprox	0	0	0	**4**	0	0	0	0
LPmid	0	**2**	0	0	0	0	0	0
IPP	0	0	0	0	0	0	0	0
JPP	0	**2**	0	0	**2**	0	0	0
Number of adhering $F4^+$E.coli per 250 µm villous length	Pig 1	Pig 2	Pig 3	Pig 4	Pig 5	Pig 6	Pig 7	Pig 8
	51	0	41	0	74	43	0	0

5.5. DISCUSSION

The present study demonstrates that the F4-specific immune response is best induced following local delivery of the F4 fimbriae in the vicinity of the jejunal Peyer's patches. Indeed, the number of F4-specific ASC in the small intestine was higher following jejunal immunisation compared to ileal, oral and colonic immunisation. Furthermore, by far the highest number of F4-specific ASC in the small intestine was detected following selective targeting of the F4 fimbriae to the jejunal Peyer's patches,

whereas selective targeting to the jejunal LP and the ileal Peyer's patches resulted in a much lower intestinal antibody response.

Oral immunisation induced a lower mucosal response than immunisation in the small intestinal lumen. This can be attributed to different factors such as (i) partial destruction of the F4 fimbriae by acid and pepsin in the stomach (Snoeck *et al.*, 2004b), digestion by enzymes released from the pancreas and/or (ii) the absence of the organised Peyer's patches in the cranial part of the small intestine (Binns and Licence, 1985). Oral immunisation, however, induced the highest systemic antibody response as evidenced by a significantly higher F4-specific serum IgG titer than following jejunal and ileal immunisation. This higher F4-specific serum IgG titer was not reflected in a higher number of IgG ASC in the small intestine. This observation and the lower mucosal immune response indicate that these F4-specific serum IgG antibodies were not mainly induced in the small intestine. It has also been described that oral immunisation with cholera toxin (CT) induces higher serum IgG than gastric immunisation (Haneberg *et al.*, 1994). Different explanations are possible: (i) Lesions in the gingiva may have been present allowing direct contact of the F4-fimbriae with the systemic immune system. Indeed, low F4-specific serum IgG responses were sometimes detected following oral immunisation of pigs lacking the F4R (unpublished results). Moreover, oral administration of F4 fimbriae to F4R-negative pigs primes the systemic immune system (Van den Broeck *et al.*, 2002); (ii) The serum response may have been induced at the nasal or larynx-associated lymphoid tissue. Indeed, nasally induced ASC home to the mucosal tissues as well as to the peripheral lymph nodes, whereas the intestinally induced ASC preferentially home to the mucosal lymphoid tissues (Quiding-Järbrink *et al.*, 1997). However, no adhesion of F4$^+$*E. coli* could be demonstrated to buccal cells, using an *in vitro* adhesion assay (Cox and Houvenaghel, 1987). Nevertheless, knowledge on the presence of F4R at the epithelium of the tonsillae linguales, pharyngeae, tubariae or palatinae is lacking.

The lower F4-specific antibody response following ileal immunisation and injection of the F4 fimbriae in the IPP demonstrates a lower importance of the continuous

ileal Peyer's patch as inductive site in 4.5 to 5 weeks old pigs in comparison with the jejunal ones. Whereas the jejunal PP (JPP) are distributed as discrete patches along the jejunum and proximal ileum, the ileal PP (IPP) occurs as a single continuous aggregation of lymphoid follicles, commencing near the ileo-caecal junction and extending cranially for a variable distance (1 to 2 m). Between these PP, structural, functional and developmental differences were observed (Binns and Licence, 1985). Whereas, the porcine JPP is definitively a secondary lymphoid organ, Andersen *et al.* (1999) demonstrated that the majority (>90%) of the IPP follicular cells are apoptosis-sensitive immature B cells, suggesting that the porcine IPP, like the sheep IPP, has a role in the generation of the primary B lymphocyte repertoire and the production of the systemic B lymphocyte pool. However, in comparison with the sheep IPP, the development of the IPP depends on antigenic stimulation, the total number of follicles increases with age and a higher number of T cells are present (Rothkötter and Pabst, 1989; Bianchi *et al.*, 1992). Therefore, Bianchi *et al.* (1992) suggested that the porcine IPP is a secondary lymphoid organ. In this study, a high number of F4-specific ASC were recovered from the IPP after immunisation in the intestinal wall or lumen. This is not consistent with their role as primary lymphoid organ. Moreover, the ileal Peyer's patch' FAE is certainly active in antigen transport. Indeed, endocytosis of F4 fimbriae (Snoeck *et al.*, submitted (b)) and particulates (Gebert *et al.*, 1994; Torché *et al.*, 2000) by ileal Peyer's patch' M cells has been demonstrated. Together these findings suggest that in addition to the generation of the primary B lymphocyte repertoire, the IPP is also involved in the induction of mucosal immunity, although its role in immune induction is less pronounced compared to the JPP.

Following injection of the F4 fimbriae in the jejunal LP, an F4-specific antibody response was induced. This demonstrates that although the LP is regarded as the effector site of the mucosal immune system, an immune response can also be induced there, at least if the antigen can successively translocate the intestinal epithelial barrier. Indeed, many antigen presenting cells (APC) are present in the porcine LP (Stokes *et al.*, 1996) which may capture and present the F4 fimbriae to T cells in the MLN. Recent studies using intestinal loop systems have clearly demonstrated the F4R-mediated endocytosis

and translocation of the F4 fimbriae by the villous epithelium and the FAE overlying the Peyer's patches (Snoeck *et al.*, submitted (b)). This can explain why a considerable F4-specific antibody response was induced following oral immunisation, since orally administered antigen will first reach the duodenum and cranial jejunum where it is probably taken up before reaching the PP. However, even a very small amount of F4 reaching the FAE may be important as the FAE can deliver the F4 fimbriae directly to the jejunal Peyer's patches, the major inductive sites. Moreover, it was remarkable finding that the F4-specific antibody response was lower following injection of the F4 fimbriae into the intestinal wall than following luminal injection. This suggests that the epithelium plays a role in the induction of the immune response. Porcine enterocytes do not express MHC class II molecules, making their role as antigen presenting cells very unlikely (Stokes *et al.*, 1996). However, proinfammatory cytokines and chemokines can be produced by the intestinal epithelium upon antigen uptake (Stadnyk, 2002). Furthermore, the F4 fimbriae may be gradually translocated across the epithelial barrier resulting in a continuously antigen release for an extended period. This may form an antigen depot and mimic slow release formulations which allow a more efficient activation of the immune system (Morein *et al.*, 1996).

The lowest antibody response was induced following immunisation in the colon. In the colon most likely no adhesion of the F4 fimbriae to the enterocytes takes place, since using an *in vitro* adhesion assay, no adhesion of F4$^+$ *E. coli* could be demonstrated to colonic villi (unpublished data) and no F4R could be identified in mucosal scrapings and contents of the colon by use of an enzyme immunoassay (Chandler *et al.*, 1994). In the spiral colon a large PP and more distally numerous 2 to 3 mm small PP can be found (Binns and Pabst, 1988). In addition to these PP, numerous lymphoglandular complexes (about 1,200 per colon) are present in the porcine colon which have an overlying epithelium containing cells compatible with M cells of the FAE (Morfitt and Pohlenz, 1989). It is likely that the small intestinal immune response following immunisation in the colon results from activation of these organised lymphoid tissues, probably following uptake of the F4 fimbriae by M cells. That the presence of the F4R on the villous

enterocytes in the small intestine is a prerequisite for the induction of a protective immune response following oral immunisation with F4 (Van den Broeck *et al.*, 1999a), whereas no receptors could be demonstrated in the colon, suggests either that sufficient F4 could be taken up by the M cells of the F4R-negative-mucosa to induce an immune response or suggests functional differences between the colonic and the small intestinal mucosal immune system. Remarkably, no F4-specific serum antibody response was induced following colonic immunisation in contrast to immunisation in the small intestine. It is not unreasonable to propose that as a result of the antigen uptake by the M cells, the immune reponse is induced in the colonic organised lymphoid tissue after which the F4-specific B cells migrate to the colonic and ileal mucosa. Following immunisation in the small intestinal lumen, the F4 will reach the systemic immune system after endocytosis by the villous enterocytes either by diffusion into the LP capillaries or following drainage to the MLN. Indeed, whereas the capillaries in the PP mucosa are non-fenestrated, the villous capillaries are highly fenestrated allowing diffusion of proteins into and out the blood (Allan and Trier, 1991). Different studies using cholera toxin (CT) or CT as adjuvant, demonstrated that colonic, colonic-rectal and rectal immunisation of mice, rats and monkeys can evoke specific antibodies in the small intestine (Haneberg *et al.*, 1994; Zhou *et al.*, 1995; Eriksson *et al.*, 1999; Shen *et al.*, 2000) with higher numbers of specific ASC in the ileum than in the jejunum (Pierce and Gowans, 1975).

In the present study the induced antibody response was studied both by enumerating the F4-specific ASC as by determining the F4-specific antibody titer in tissue extracts of the jejunum. Findings demonstrate that the antibody titers in the jejunal mucosa are not always correlated with the number of ASC found there. The lack of correlation can have several reasons: (i) the antibody response is localised and not evenly distributed along the small intestine, as demonstrated by the higher correlation found when the proximal and mid-jejunum were regarded as a whole. Indeed, even the antibody titer in the small tissue pieces, isolated from 2 jejunal segments 5 cm apart from each other, revealed sometimes differences in antibody titer. (ii) The lack of correlation most likely results from diffusion of serum antibodies in the LP via the highly fenestrated

villous capillaries (Allan and Trier, 1991). Indeed, there was a high correlation between the F4-specific IgG titer in the jejunal mucosa and the serum IgG titer (r = 0.987). However, also IgM and IgA can diffuse in the mucosa. In the pig it has been demonstrated for example that 22.7-29.5% of IgA in the gut lymph comes from serum, versus 77.3 to 70.5% from local production in the intestine. Conversly, 31% of the total plasma IgA originates from local intestinal synthesis, reaching the blood via mesenteric lymph (Vaerman *et al.*, 1997). Nevertheless, it is generally assumed that locally produced antibodies are of major importance in terms of protection in the intestine compared to the serum derived antibodies.

5.6. CONCLUSION

The present study demonstrates that the jejunal Peyer's patches are the major inductive sites for the F4-specific intestinal immune response, whereas the lamina propria and the ileal Peyer's patch are of less importance as inductive sites. Therefore, selective targeting of the F4 fimbriae to these jejunal Peyer's patches by delivery vehicles would be the most effective way of vaccination and may reduce the number of doses needed for primary vaccination.

5.7. ACKNOWLEDGEMENTS

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CHAPTER 6

SPECIFIC ADHESION OF F4-FIMBRIAE TO AND ENDOCYTOSIS BY VILLOUS AND DOME EPITHELIA IN F4-RECEPTOR POSITIVE PIGS

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6.1. ABSTRACT

Orally administered F4 (K88) fimbriae induce mucosal immune responses in F4R+ pigs, whereas no F4-specific antibody secreting cells can be found in the intestine of F4R- pigs. In the present study, we investigated the *in vivo* adhesion of the isolated F4 fimbriae (F4) to villous epithelium and the follicle-associated-epithelium (FAE) overlying the Peyer's patches (PP) of the jejunum and ileum in 1 F4R- and 9 F4R+ pigs using jejunal loops with or without a discrete PP (JPP and jejunal loops, respectively) and loops in the region of the continous PP of the ileum (ileal loops). Immunohistochemical analysis of the sections of the different loops indicated that F4 did not adhere to the intestinal epithelium in the F4R- pig. On the other hand, in the F4R+ pigs, F4 strongly adhered to the epithelium of the villi in the jejunal loops and to the villi at the mesenteric and antimesenteric side in the JPP and IPP loops. Furthermore, F4 strongly bound to both the follicle-associated-enterocytes as well as the M cells of the FAE in JPP and IPP loops. Moreover, following F4R binding, endocytosis and transcytosis of the F4 across the epithelium was observed after which F4 was released in the small intestinal lamina propria and dome region of the jejunal and ileal PP. These observations may explain the success of F4 as immunogen following oral administration in F4R+ pigs, as the binding of F4 to the F4R causes its endocytosis and translocation across the epithelium. Once delivered under the epithelium it can be taken up by professional antigen presenting cells which activate the gut-associated immune system.

6.2. INTRODUCTION

F4+ *Escherichia coli* are an important cause of diarrhoea in recently weaned pigs (Hampson, 1994). It has been demonstrated that oral administration of isolated F4 (K88) fimbriae (F4) to pigs actively induce mucosal and protective immune responses against F4+ *E. coli* infection (Van den Broeck *et al.*, 1999a). However, the presence of F4-specific receptors (F4R) on the brush borders of the villous enterocytes is a prerequisite for the induction of this immune response, as in pigs lacking these F4R (F4R- pigs) no F4-specific antibody-secreting cells (ASC) can be induced in the intestine following oral administration. Consequently, it has been hypothesised that F4 binding to these F4R is

crucial to obtain activation of the immune system upon oral administration. However, data about the *in vivo* binding of isolated F4 to intestinal epithelial cells in F4R+ and F4R- pigs is lacking. Moreover, no data are available about distribution of the binding along the small intestine (i.e. the jejunum versus ileum). Activation of the gut-associated immune system implies that the antigen can penetrate into or through the intestinal epithelium to reach the immune cells. At this moment, it is not known whether the binding to the F4R results in endocytosis of the fimbriae in the epithelium and/or translocation through the epithelial layer. Furthermore, the putative role of the M cells, present in the follicle-associated-epithelium (FAE) overlying the Peyer's patches (PP), in the F4 immunisation model is not clear.

In the present study, we analysed the *in vivo* adhesion of isolated F4 to the intestinal epithelium of the jejunum and ileum of F4R+ pigs and one F4R- pig after 5 min, 15 min and 1 h. We further examined whether, upon binding to the F4R, F4 is endocytosed in the epithelial cells (enterocytes and/or M cells) and if so whether this results in transcytosis of F4 across the epithelium. Insights in how the F4 penetrates and translocates the epithelial layer of the gut are essential in understanding the immunogenicity of F4 via the oral route (Van den Broeck *et al.*, 1999a) and therefore could give valuable information for the development of other oral vaccines.

6.3. MATERIAL AND METHODS

6.3.1. Isolation of F4

The F4 was isolated from the bacteria by mechanical shearing as previously described (Van den Broeck *et al.*, 1999c). The protein concentration of the isolated F4 solution was determined using the bicinchoninic acid protein assay kit (Sigma-Aldrich, Bornem, Belgium). The purity was assessed by electrophoresis on a SDS-12% polyacrylamide slab gel, followed by analysis of the Coomassie stained gel using the gel analysis software, Image Master 1D® (Amersham Pharmasia biotech, Newcastle upon Tyne, England), so that the concentration of the F4 in the solution could be calculated.

6.3.2. Labeling of F4 with fluorescein

The F4 was purified from the isolated F4 solution by anion exchange chromatography using a Bio-scale Q2 column (BIO-RAD Laboratories, Nazareth, Belgium) as previously described (Van den Broeck *et al.*, 1999c). The purified F4 was labeled with 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS, 480 Da) using the fluorescein labeling kit (Roche Diagnostics GmbH, Mannheim, Germany). The molar reaction ratio F4:FLUOS used was 1:10. Remaining non-reacted-FLUOS was blocked by adding 1:100 of 0.1 M Glycin in PBS (150mM, pH 7.4) to reach a final concentration of 1mM Glycin (incubation 1 h at 18°C), which was subsequently removed by dialysis against PBS using a membrane with a cut off of 14 kDa. Before use, the binding of the FLUOS-labeled-F4 to the F4R on villous brush border enterocytes was tested *in vitro*. Therefore, 30 µg FLUOS-labeled-F4 was incubated with 10 villi from $F4R^+$ and $F4R^-$ pigs, isolated as previously described (Van den Broeck *et al.*, 1999c), in a volume of 50 µl Krebs-Henseleit buffer (pH 7.4) for 1 h at room temperature (RT) while being gently shaken. The villi were washed 3 times with 0.5 ml Krebs-Henseleit buffer, followed by evaluation using fluorescence microscopy at a magnification of 600. Furthermore, the polymeric nature of the FLUOS-labeled F4 was compared with unlabeled F4 by electrophoresis on a SDS-12% polyacrylamide slab gel without prior boiling of the samples, followed by immunoblotting using the F4-specific MAb (IMM01, Van der Stede *et al.*, 2002).

6.3.4. Animals and surgical techniques

Experimental procedures and animal management procedures were undertaken in accordance with the requirements of the animal care and ethics committee of Faculty of Veterinary Medicine, Ghent University, Belgium (EC 98/14). Ten pigs (Belgian Landrace x English Landrace, Dutch Landrace, 3 litters) seronegative for antibodies against F4 as determined by ELISA, were weaned at the age of 4 weeks. Subsequently, they were housed in isolation units where they were fed at libitum. To prevent outbreaks of *E. coli* infections during the weaning period, all animals were orally treated during 3 successive days with colistine (150, 000 U/kg body weight; Colivet, Prodivet Pharmaceuticals, Eynatten, Belgium) from 1 day before weaning.

Five days postweaning, the pigs were anaesthetized intramuscularly with tiletamine and zolazepam (Zoletil 100; Virbac S.A., Carros, France) supplemented with 2% xylazine (Xyl-M®; VMD, Arendonk, Belgium) (0.22 ml/kg) after an overnight fast. In 6 pigs (pig nº 1 to 6), 3 jejunal gut loops without PP (jejunal loops), 3 jejunal gut loops each including a discrete PP (JPP loops) and 3 gut loops in the region of the continuous PP of the ileum (IPP loops) were created following laparotomy as previously described (Moon *et al.*, 1966). Each loop was 5 cm long and loops were separated from each other by an interloop segment of 5 cm. Care was taken to minimize surgical trauma and to maintain an adequate blood supply to the ligated segments. Buffer (5 ml PBS containing 1mM $CaCl_2$ and 1mM $MgCl_2$), F4 (3 mg in 5 ml buffer) and FLUOS-labeled-F4 (3 mg in 5 ml buffer) were each injected into the lumen of a jejunal loop, a JPP loop and an IPP loop with a 26 gauge needle. Subsequently, the small intestine was returned to the abdominal cavity and the abdomen was closed. General anaesthesia was maintained for 1 h or 15 min (pig nº 1 to 3 and pig nº 4 to 6, respectively), allowing *in vivo* adhesion of F4 to the villous brush borders. To examine early adhesion of F4 fimbriae, in 3 additional pigs (pig nº 7 to 9) F4 or buffer were injected in 2 IPP loops, as described above, and incubated for 5 min. No JPP and jejunal loops were created in these 3 pigs, as no differences in F4 adhesion were found between JPP, IPP and jejunal loops after 15 min and 1 h incubation, as described in the results. Finally, the animals were euthanised after an incubation of 5 min, 15 min or 1 h with an intravenous injection of an overdose of pentobarbital (24 mg/kg; Nembutal, Sanofi Santé Animale, Brussels, Belgium) and the following samples were collected: (i) intestinal contents of both the jejunum as well as ileum and (ii) blood for the determination of F4-specific IgM, IgA and IgG titers in intestinal contents and serum; (iii) samples for immunohistochemical analysis; (iii) jejunal villi for the determination of the F4R status.

One pig was selected as an F4R- control animal (pig nº 10) by biopsy of the small intestine and verification of the F4R- status in the *in vitro* villous adhesion assay. In this pig, 6 jejunal loops, 6 JPP loops and 6 IPP loops were created. Three of these 6 loops were injected with buffer, F4 or FLUOS-labeled F4 as described above. Subsequently, the small intestine was returned to the abdominal cavity after which the abdomen was closed. After

40 min, the abdomen was reopened and the remaining 3 loops were injected with buffer, F4 and FLUOS-labeled F4, respectively. Subsequently, the intestine was returned into the abdominal cavity and the abdomen was surgically closed. Fifteen min later the pig was euthanised and the samples were collected as described above.

6.3.5. F4-specific IgM, IgA and IgG in intestinal contents and serum

The intestinal contents of jejunum and ileum were two-fold diluted in PBS supplemented with 20% fetal calf serum, penicillin (100 IU/ml), streptomycin (100 µg/ml) and 0.2% (v/v) Tween®20. Following incubation for 30 min at 56°C, the samples were centrifuged at 4°C and 9500 x *g* during 10 min. The supernatant was centrifuged once again and stored at -70°C until analysis.

Blood was collected from the jugular vein. After 18 h incubation at RT, serum was collected, inactivated at 56°C during 30 min and subsequently treated with kaolin (Sigma-Aldrich) to decrease the background reading in ELISA (Van den Broeck *et al.*, 1999a). Subsequently, the serum was diluted in ELISA dilution buffer [PBS + 0.05% (v/v) Tween®20 (Merck, Hohenbrunn, Germany) + 3% (w/v) bovine serum albumin (BSA; Sigma-Aldrich)] to obtain a final serum dilution of 1/10.

For detection of F4-specific antibodies in the intestinal contents and serum, the indirect ELISA described by Van der Stede *et al.* (2003) was used. As negative control the serum of an SPF-pig and the intestinal contents of a seronegative 12-week old pig were used and the obtained OD_{405} value after 30 min incubation was used as cutoff value. The serum cutoff values were 0.25, 0.33 and 0.35 for F4-specific IgM, IgA and IgG, respectively and the cutoff values for the intestinal contents were 0.29, 0.31 and 0.29 for IgM, IgA and IgG, respectively. The antibody titer was determined as the inverse of the highest dilution that still had an OD_{405} higher than the cutoff value.

6.3.6. *In vitro* villous adhesion assay for F4R characterisation

In order to determine the presence or absence of F4R on the small intestinal villous enterocytes, an *in vitro* villous-adhesion assay was performed as described previously (Van den Broeck *et al.*, 1999c). Adhesion of less than 5, less than 30 and more than 30

bacteria per 250 µm villous brush border length was noted as negative, weak positive, and strong positive, respectively (Cox and Houvenaghel, 1993).

6.3.7. Antibodies and reagents for immunohistochemical analysis

The following antibodies were used as primary antibodies during immunohistochemistry: anti-cytokeratin peptide 18 (clone CY-90, IgG1, Sigma-Aldrich); anti-epidermal growth factor receptor (EGFR; clone 29.1, IgG1, Sigma-Aldrich); anti-F4 protein (IMM01, IgG, Van der Stede *et al.*, 2002); anti-SWC3 (undiluted hybridoma supernatant, 74-22-15). The following antibodies were used as secondary antibodies during immunohistochemistry: fluorescein isothiocyanante (FITC)-labeled sheep anti-mouse IgG (Sigma-Aldrich); Texas Red®-X-labeled goat anti mouse IgG (Molecular Probes, Leiden, The Netherlands); Biotin-conjugated rabbit anti-mouse IgG (Dako, Prosan, Merelbeke, Belgium). Peroxidase-conjugated-streptavidin was purchased from Dako (Prosan).

6.3.8. Immunohistochemistry

Immediately after euthanasia, the loops were excised, opened at both ends and flushed with cold PBS containing 1mM $CaCl_2$ and 1mM $MgCl_2$. Subsequently, the loops were cut into 2. The first part was embedded in 2% (w/v) methocel (Fluka, Bornem, Belgium) in water and immediately frozen in liquid nitrogen. From the frozen tissue samples, approximately 8 µm thick cryosections were cut and mounted on 3-aminopropyl-triethoxysilane (APES; Sigma-Aldrich) coated glass slides. After drying for 4 h at 60°C, the sections were fixed in acetone for 10 min at -20°C and stored at -70°C. The second part of the loop was opened longitudinally at the mesenteric side and fixed in 3.5% (v/v) formaldehyde in PBS for 18 h. After dehydrating the tissue, it was embedded in paraffin after which 5 µm thick sections were cut and mounted on APES-coated glass slides. However this was not done for the loops injected with FLUOS-labeled F4.

Cryosections were air-dried during 1 h. Subsequently, they were washed in PBS for 5 min after which they were incubated with 10% (v/v) animal serum (goat or sheep serum depending on the species of the secondary antibody used) in PBS for 30 min at 37°C to decrease background staining. Subsequently, the sections were sequentially incubated

with the primary antibody diluted in PBS and with the FITC- or TexRed®-conjugated secondary anti-mouse antibody (in PBS) supplemented with 5% pig serum, both during 1 h at 37°C. The sections were mounted in glycerol containing 0.223 M 1,4-diazobicyclo-(2,2,2)-octane (DABCO; Sigma-Aldrich) to counter photobleaching. When a double staining was performed, the sections were first blocked during 30 min at 37°C with 10% animal serum of both species from which the first as well as the second secondary antibody were derived. Subsequently, the following antibodies were sequentially applied and incubated for 1 h at 37°C: the first primary antibody, the first secondary antibody, the second primary antibody and the second secondary antibody.

Paraffin sections were deparaffinised in xylene, rehydrated in a series of ethanol dilutions and transferred to distilled water. Pre-treatment of the sections with 0.1% (w/v) protease (Sigma-Aldrich) in PBS for 10 min at 37°C enhanced the staining intensity. Subsequently, the sections were incubated with 3% (v/v) H_2O_2 in distilled water for 5 min at RT, followed by 10% (v/v) rabbit serum in PBS for 30 min at 37°C to decrease the endogenous peroxidase activity and background staining, respectively. Subsequently, the F4-specific MAb in dilution buffer [PBS + 2% (w/v) BSA] was added and incubated for 1 h. Thereafter, the sections were sequentially incubated with the biotin-conjugated secondary anti-mouse antibody for 1 h and with the peroxidase-conjugated streptavidin (Dako, Prosan) for 45 min, both at 37°C. Finally, the peroxidase label was developed in a freshly prepared solution of diaminobenzidin (DAB; Dako, Prosan) and Mayer's hematoxylin was applied for 10 s as nuclear counterstain.

All incubations were carried out in a humidified environment, and between the incubation steps the slides were washed 3 times with PBS for 5 min each. Controls omitting the primary antibodies, the secondary antibodies, and/or the streptavidin-peroxidase-conjugate were performed.

6.3.9. Analysis of binding to and uptake by the epithelium

The *in vivo* adhesion of F4 to the epithelium was examined after 5 min (IPP loops), 15 min (jejunal, JPP and IPP loops) and 1 h (jejunal, JPP and IPP loops). Adhesion after 15 min and 1 h was evaluated light microscopically on the paraffin sections at a

magnification of 300, followed by verification on cryosections stained with the F4-specific MAb. Adhesion after 5 min was only evaluated on cryosections, since less adhesion was expected after 5 min than after 15 min and 1 h and fluorescence staining on the cryosections is more sensitive than the peroxidase staining on the paraffin sections. For each loop, three sections (at the beginning, the middle and the end) were examined. In jejunal loops, the number of villi to which F4 adhered was counted, whereas in JPP and the IPP loops, villi of the antimesenteric side (i.e. overlying the PP), villi of the mesenteric side (i.e. without PP) and domes with FAE to which F4 adhered were counted separately. Only longitudinally cut villi and/or obviously distinguishable dome areas above the PP were taken into account.

Cryosections were further used to analyse the uptake of the F4 by the villous epithelium and FAE by conventional fluorescence microscopy and confocal laser scanning microscopy (CLSM).

6.3.10. Statistics

Statistical analysis was performed using the software package SPSS version 11.0. The data were tested fore normal distribution using the Kolmogorov-Smirnov test and homogeneity of variance using the Levene's test. Differences in percentage F4 adhesion to the villi and FAE between the different incubation periods and between the different intestinal segments were tested for statistical significance using One-Way Anova or T-test for normally and non-normally distributed data, respectively.

6.4. RESULTS

6.4.1. *In vitro* villous adhesion assay for F4R characterisation

Nine pigs expressed the F4R on the villous brush border. Eight pigs were strong F4R positive, whereas two pigs (nº 5 and 8) were weak F4R positive. Pig nº 10 was F4R negative (Fig. 6.1.).

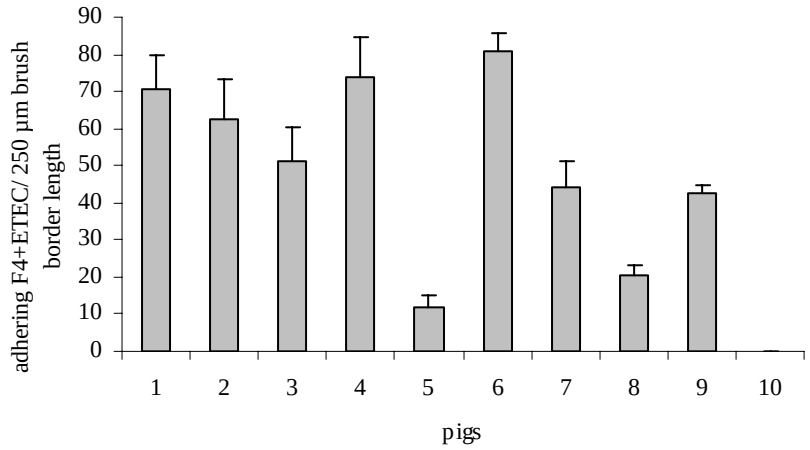

Fig. 6.1. Number of adhering F4+ETEC per 250 µm length of intestinal villous brush border of the 10 individual pigs (mean ± SD, n =3).

6.4.2. F4-specific IgM, IgA and IgG in serum and intestinal contents

As the F4 adhesion can be influenced by the presence of F4-specific antibodies in the intestinal lumen, the F4-specific IgM, IgA and IgG titer were determined in serum and intestinal contents of the jejunum and ileum. In 4 and 6 pigs, respectively, a weak F4-specific serum IgM and IgG titer (ranging from 15 to 40) was found, obtained by maternal and colostral antibodies (Table 6.1). In none of the pigs, F4-specific serum IgA could be found. In 2 pigs, n° 3 and 4, substantial F4-specific IgM and/or IgA were noticed in the intestinal contents and not in the serum, indicating an active antibody production in the intestine.

Table 6.1. F4-specific IgM, IgA and IgG titer in the intestinal contents of the jejunum and the ileum and serum of the pigs on the moment of euthanasia.
Titers above the background level are indicated in bold. -, not determined.

Pig	IgM			IgA			IgG		
	jejunum	ileum	serum	jejunum	ileum	serum	jejunum	ileum	serum
1	2	2	10	2	2	10	2	2	**15**
2	2	2	10	2	2	10	2	2	10
3	2	**8**	10	2	2	10	2	2	**20**
4	-	**256**	10	-	**64**	10	2	2	**15**
5	2	2	**15**	2	2	10	2	2	**15**
6	**4**	**3**	10	**3**	2	10	2	2	**15**
7	2	2	**15**	**3**	**3**	10	2	2	10
8	2	2	**40**	2	2	10	2	2	**20**
9	2	2	**20**	**3**	**3**	10	2	2	10
10	**4**	**3**	10	2	2	10	2	2	10

6.4.3. Adhesion of F4 in the jejunum and ileum

The *in vivo* adhesion of F4 to the epithelium was examined after 5 min (IPP loops), 15 min (jejunal, JPP and IPP loops) and 1 h (jejunal, JPP and IPP loops). The shortest incubation period was done to investigate whether the F4 already adhered to the villi of the mesenteric and antimesenteric side and the FAE after 5 min. However, this was only examined in IPP loops, as no differences in F4 adhesion was found between jejunal, JPP and IPP loops after 15 min and 1 h, as further described. The F4 adhesion was analysed by counting the number of positive villi and FAE on 3 sections made of each loop. The results for F4 adhesion after 5 min are given in Table 6.2 which is representative for mode of

analysis of all loops. In all sections examined, the positive villi and FAE were equally distributed, and no differences were noted between the 3 examined sections of each loop. In all animals, section of the control loops incubated with PBS remained negative.

Table 6.2. Example of analysis of the F4 adhesion to the intestinal epithelium in the different loops.
Number and percentage villi and FAE positive for F4 adhesion in the IPP loops of F4R+ pigs after 5 min *in vivo* incubation [positive/total, percentage %]. The data represent the total results of three sections per segment.

Pig	IPP		
	Villi		FAE
	Mesenteric side	Antimesenteric side	Antimesenteric side
7	69/69 100%	66/66 100%	22/22 100%
8	141/141 100%	135/135 100%	10/14 71%
9	0/130 0%	30/192 16%	6/18 33%
Mean %	**67%**	**72%**	**68%**

F4 strongly adhered to the villous epithelium of F4R± pigs (Fig. 6.2). In the JPP and IPP loops, the F4 adhesion to the villi at the mesenteric side and the antimesenteric side was examined to elucidate whether the F4R expression differed between these to sides. Since no differences were found between adhesion to villi at the mesenteric and antimesenteric side, no further division between villi at the different sides of the mesenterium was made in the description of the results. No significant differences in F4 adhesion to the villous epithelium were found between the different incubation periods. However, the intensity of the staining and the thickness of the F4 band at the brush border were less after 5 min incubation. Furthermore, no differences in adhesion to the villous epithelium were found between jejunal, JPP and IPP loops. In jejunal loops, an average of 96% (ranging of 87-100%) and 100% of all the examined villi were positive after 15 min and 1 h, respectively. In the JPP loops, F4 adhered to an average of 91% (ranging from 57-100%) and 98% (ranging from 92-100%) of the villi after 15 min and 1 h, respectively. In the IPP loops, an average of 69% (0-100%), 99% (92-100%) and 93% (87-100%) of the villi showed a positive reaction, after 5 min, 15 min and 1 h,

respectively. Adhesion of the F4 was detected on the tip and both sides of all villi examined. On the contrary, the crypt cells never showed a positive reaction (Fig. 6.2).

F4 also strongly adhered to the FAE of F4R± pigs (Fig. 6.2). Compared to the percentage positive villi, the percentage positive FAE was not significantly different. In the JPP loops, 83% (50-100%) and 72% (16-100%) of the FAE was positive after 15 min and 1 h, respectively. These percentages were similar to those obtained in the IPP loops, namely 68% (33-100%), 82% (46-100%) and 89% (69-100%) after 5 min, 15 min and 1 h, respectively. As can be expected, no significant differences could be found in F4 adhesion to the FAE between the different incubation periods.

In 3 pigs, [n° 3 (incubated for 1h), n° 5 (incubated for 15 min) and n° 9 (incubated for 5 min)] less F4 adhesion was observed than in the other pigs. In pig n° 3 less adhesion was noticed to the FAE of the jejunal and ileal PP (16 and 69%, respectively), whereas the F4 strongly adhered to the villous epithelium (87-100%). In pig n° 5, F4 adhered more weakly compared to the other pigs to the villi (57-87%) and the FAE in the JPP (17%) and IPP loop (46%). In pig n° 9, weak to no adhesion to the villi (0-16%) and the FAE (33%) was observed. Whereas pig n° 5 was weak F4R+, this was not the case for pigs n° 3 and 9 (Fig 6.1). Furthermore, pig n° 8 was also weak F4R+, but nevertheless showed strong F4 adhesion. In pig n° 3 a substantial F4-specific IgM titer was found in the luminal content of the ileum but not in the content of the jejunum (Table 6.1). However, in pig n° 4 (incubated for 15 min) with the highest F4-specific antibodies in the intestinal contents, strong F4 adhesion was observed, indicating that the amount of F4 injected in the loops was high enough to overcome these antibodies. In contrast to all the other pigs, large amounts of intestinal content were present in the loop of pig n° 9, even after the overnight fast.

In the F4R- pig (n° 10), F4 adhered neither to the villi nor to the FAE after 15 min and 1 h incubation (Fig. 6.2.).

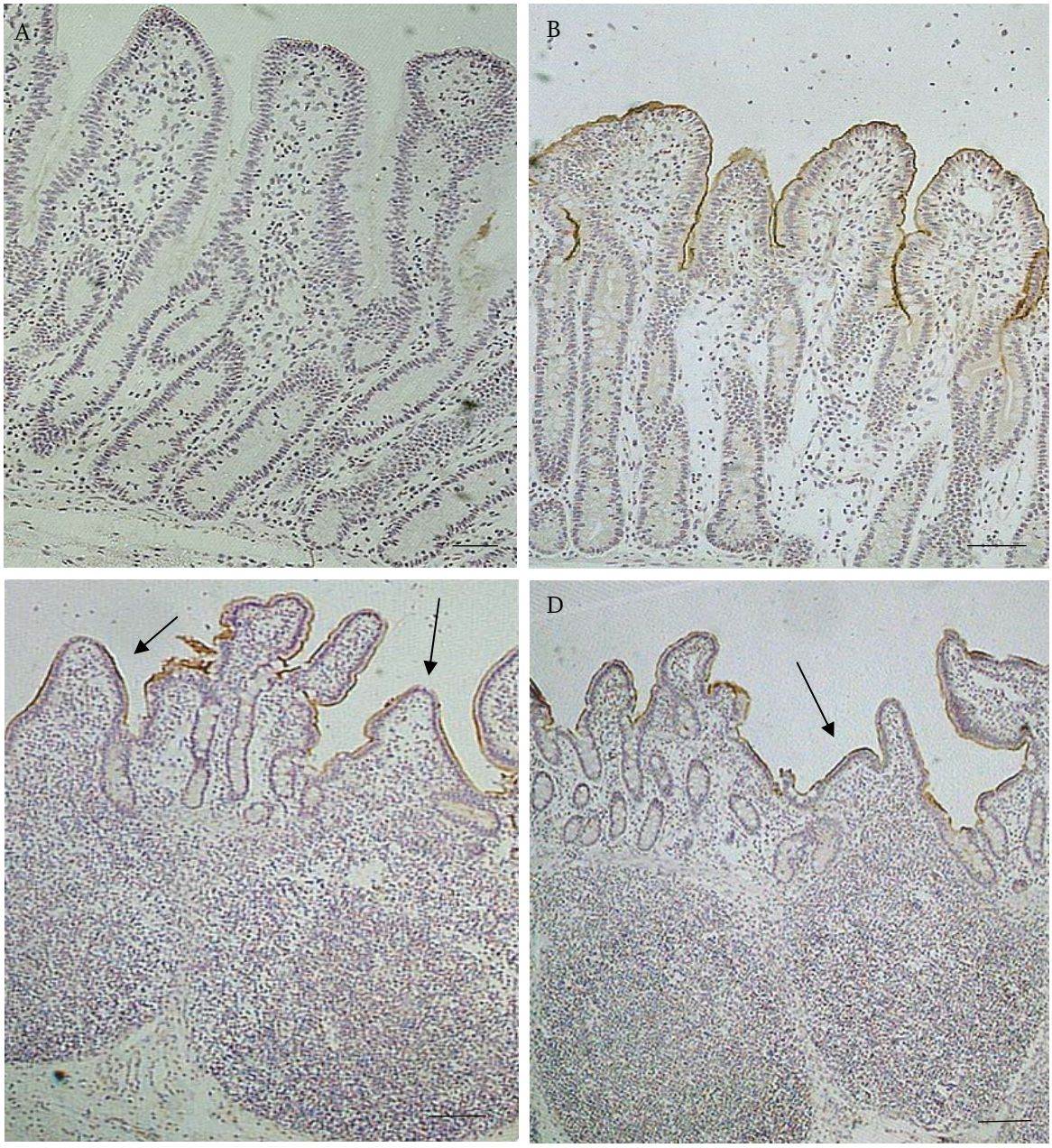

Fig. 6.2. Paraffin section of the jejunum of an F4R-pig after 1 h in vivo incubation with isolated F4 (A); paraffin section of the jejunum (B), the jejunal Peyer's patch (C) and the ileal Peyer's patch (D) of an F4R+pig after 15 min incubation with isolated F4. The F4 did not adhere to the villi of the F4R-pig. In the F4R+pig, the sides and the tip of the villi were positive whereas the crypts remain negative. F4 adhered to the FAE of both the jejunal as ileal Peyer's patch (arrows); bar 100 μm (A, B) and 200 μm (C,D).

6.4.4. FLUOS-labeled F4 fimbriae still bind to the F4R

Before injection of the FLUOS-labeled F4 into loops, its binding properties to the F4R were determined *in vitro*. Incubation with villi of F4R+ and F4R- pigs demonstrated a green fluorescent band at the villous brush border of F4R+ villi, which was absent on F4R- villi (data not shown). The polymeric nature of the F4 was maintained following labeling with FLUOS as demonstrated on western blotting (Fig. 6.3). The staining intensity of the FLUOS-labeled F4 was reduced in comparison with the non-labeled F4, most likely due to a diminished accessibility of the epitope for the F4-specific MAb IMM01 caused by the FLUOS-label.

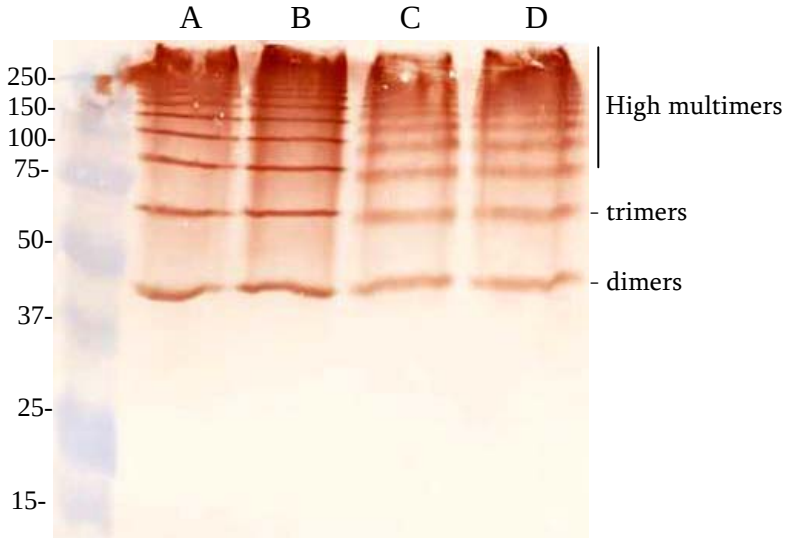

Fig. 6.3. Evaluation of the polymeric nature of FLUOS-labeled F4 (lane C and D) compared to non-labeled F4 (lane A and B) by immunoblotting. Dimers, trimers and higher multimeric forms of the major subunit of the F4 fimbriae are detected. Molecular weight is indicated.

6.4.5. Endocytosis and translocation of F4 by villous enterocytes

Endocytosis of F4 by the epithelial cell was studied on cryosections of loops incubated with F4 and FLUOS-labeled F4. FLUOS-labeled F4 was used as it can be expected that F4 will be partially degraded following uptake into the cell and may therefore be difficult to visualise using F4-specific MAb. On the other hand, the labeling of F4 by FLUOS may have an influence on the uptake and transport of the F4 through the

epithelial cells. Therefore, endocytosis of F4 was studied using both F4 as well as FLUOS-labeled F4.

The binding of FLUOS-labeled F4 (and non-labeled F4) to and its endocytosis by the villous enterocytes was studied by (dual) immunohistochemical staining of (F4 and) cytokeratin 18 or EGFR. Cytokeratin 18 is exclusively present in epithelial cells (Gebert *et al.*, 1994). Whereas the enterocytes are weakly stained, the thin cytoplasmic rim of the goblet cells is intensively stained by the anti-cytokeratin 18 MAb. The anti-EGFR MAb binds to a carbohydrate residue on the external portion of the EGFR that is shared by blood group type A determinants on various glycoproteins and glycolipids. King and Kelly (1991) demonstrated that these blood group type A determinants are expressed on glycoproteins and glycolipids at the apical membrane of porcine enterocytes. These stainings demonstrated that F4 bound to the enterocytes, but not to goblet cells. In the 5 min incubated loops, no F4 was detected intracellularly. However, in the 15 min incubated loops, F4 could be demonstrated in the cytoplasm of the enterocytes, beneath the epithelial layer and even in the core of the villus (Fig. 6.4. and 6.5.), demonstrating endocytosis and translocation through the enterocytes. To elucidate whether the F4 was captured by immune cells following translocation through the epithelium, the cryosections were stained for SWC3$^+$ cells. The SWC3-specific antibody stains a heterogenous population of cells of myeloid origin present in the porcine small intestinal LP, including mast cells, non-professional antigen presenting MHCII$^+$ eosinophils, and professional antigen presenting MHCII$^+$ macrophages and dendritic cells (Stokes *et al.*, 1996; Haverson *et al.*, 2000). Indeed, in the core of the villus, the F4 could occasionally be identified in SWC3$^+$ cells (Fig. 6.6.). Furthermore, F4 could also rarely be identified in SWC3$^-$ cells. No difference could be found between the 1 h and 15 min incubated loops.

No differences were found between non-labeled F4 and FLUOS-labeled F4 except for the fact that staining intensity was less in the case of non-labeled F4 and therefore endocytosis and translocation were less obvious in loops incubated with the F4 compared to the FLUOS-labeled F4.

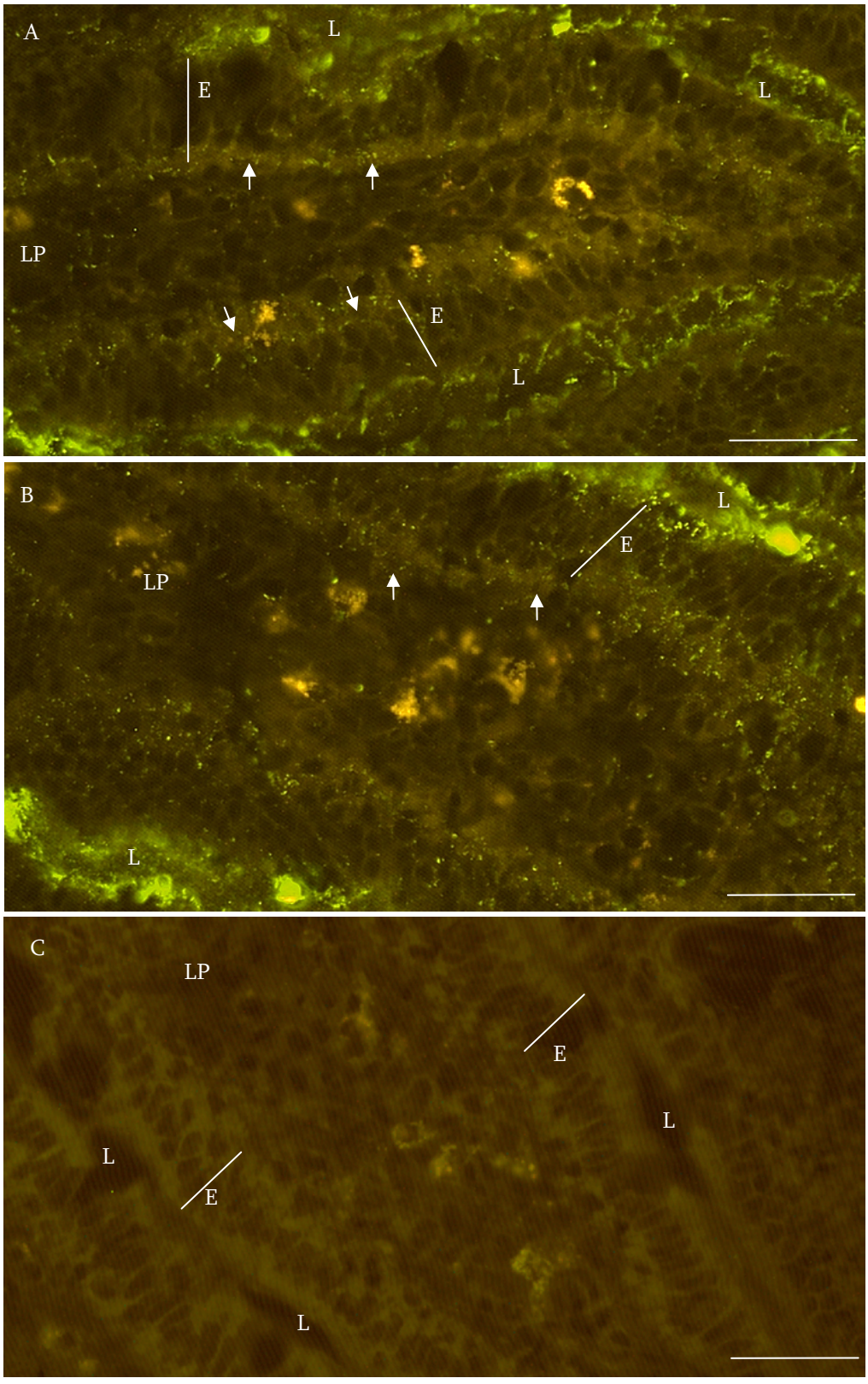

Fig. 6.4. Cryosection of the jejunum of an F4R+ pig after 1 h (A) and 15 min (B) and of the F4R- pig after 1 h *in vivo* incubation with F4 (C). Immunohistochemical detection of F4 by the F4-specific MAb (*green* fluorescence). F4 was clearly present in the enterocytes, beneath the nucleus of the enterocytes (white arrows) and in the core of the villus in the F4R+ pigs, whereas in the F4R- pig no F4 could be identified. The yellow cells represent autofluorescent granulocytes. L= lumen, LP= lamina propria, the width of the epithelial layer (E) has been indicated. Bars, 50 µm.

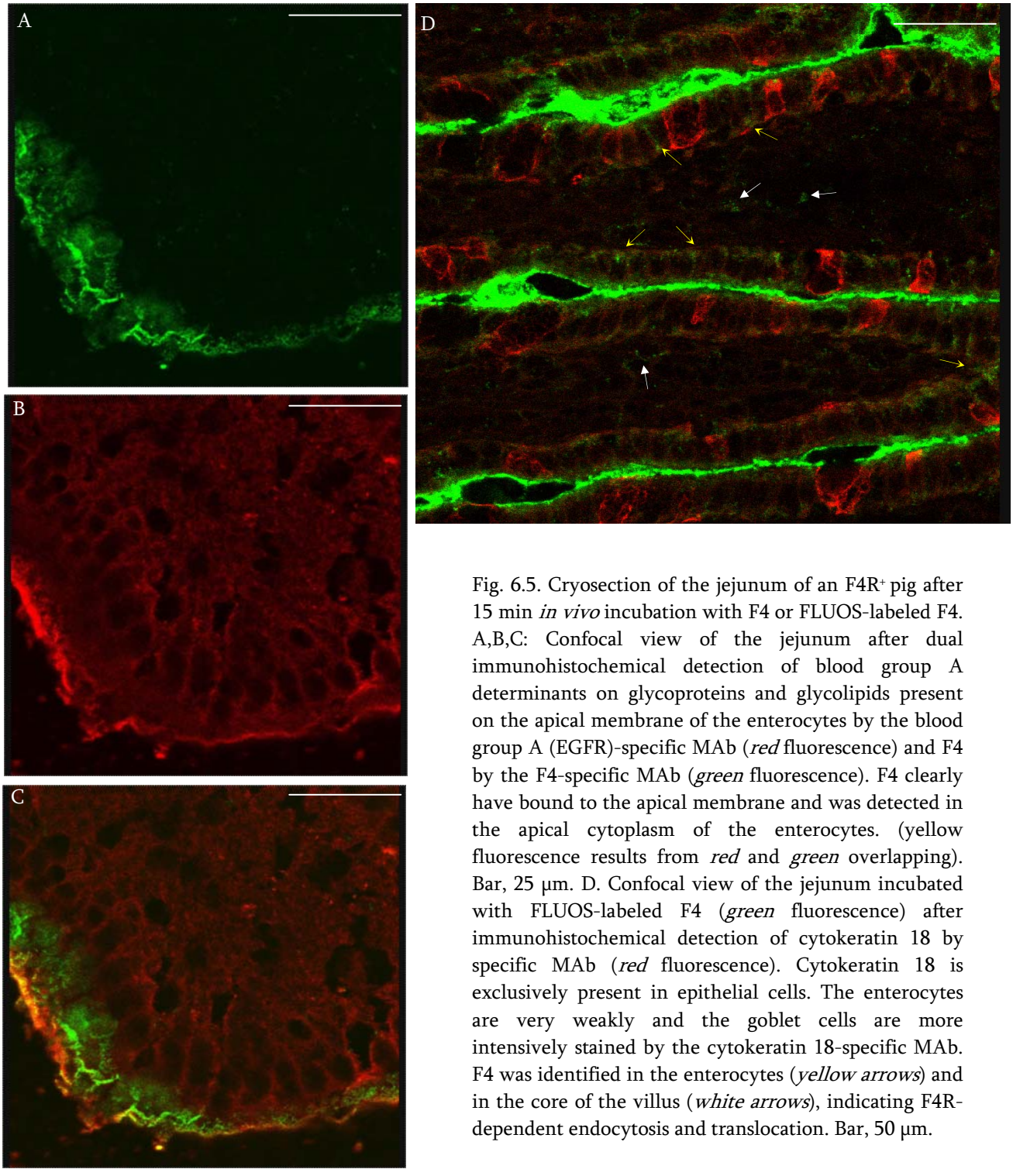

Fig. 6.5. Cryosection of the jejunum of an F4R+ pig after 15 min *in vivo* incubation with F4 or FLUOS-labeled F4. A,B,C: Confocal view of the jejunum after dual immunohistochemical detection of blood group A determinants on glycoproteins and glycolipids present on the apical membrane of the enterocytes by the blood group A (EGFR)-specific MAb (*red* fluorescence) and F4 by the F4-specific MAb (*green* fluorescence). F4 clearly have bound to the apical membrane and was detected in the apical cytoplasm of the enterocytes. (yellow fluorescence results from *red* and *green* overlapping). Bar, 25 µm. D. Confocal view of the jejunum incubated with FLUOS-labeled F4 (*green* fluorescence) after immunohistochemical detection of cytokeratin 18 by specific MAb (*red* fluorescence). Cytokeratin 18 is exclusively present in epithelial cells. The enterocytes are very weakly and the goblet cells are more intensively stained by the cytokeratin 18-specific MAb. F4 was identified in the enterocytes (*yellow arrows*) and in the core of the villus (*white arrows*), indicating F4R-dependent endocytosis and translocation. Bar, 50 µm.

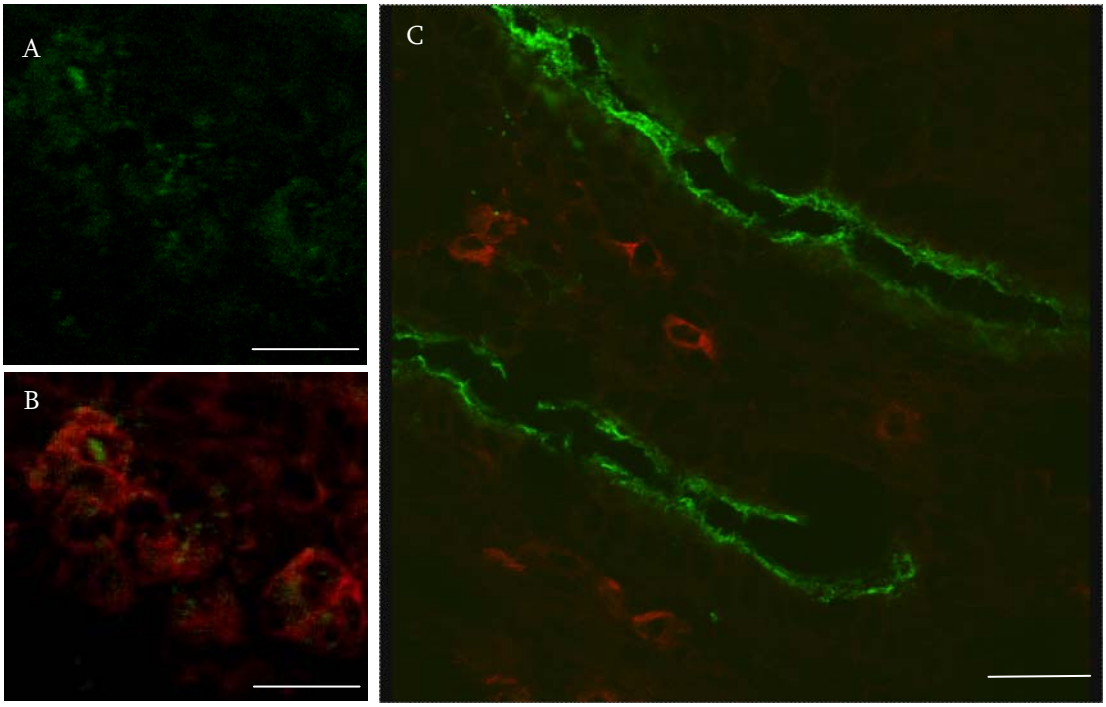

Fig. 6.6. Confocal view of the jejunum of an F4R+ pig incubated with FLUOS-labeled F4 (*green* fluorescence) after immunohistochemical detection of SWC3 by specific MAb (*red* fluorescence). F4 was identified in the cytoplasm of SWC3+ cells (A,B) and rarely in the SWC3- cells (C). Bars, 17 µm (A, B), 20 µm (C).

6.4.6. Endocytosis of F4 by follicle-associated enterocytes and M cells

Analysis of the FLUOS-labeled F4 binding to the FAE by immunohistochemical staining of cytokeratin 18, revealed that F4 bound to both the follicle-associated enterocytes as well as M cells of both the jejunal and ileal PP (Fig. 6.7.). M cells were identified using the anti-cytokeratin 18 MAb; the cytoplasm of M cells contains a network of intensely stained cytokeratin 18 surrounding the nucleus and forming a dense plaque in the most apical cytoplasm, whereas the enterocytes are weakly stained. However, F4 did not bind to 5% of the M cells present in the FAE (data not shown). Whereas in the 5 min incubated loops, F4 could not be identified in the cells of the FAE, endocytosis of F4 by both the follicle-associated enterocytes as M cells could be observed in the 15 min incubated loops (Fig. 6.8 and 6.9). Furthermore, F4 could be identified in the dome region of the PP, demonstrating transcytosis of F4 by the FAE (Fig. 6.8). However, transcytosis of F4 by the M cells could not be determined by certainty. No difference in endocytosis and translocation activity could be noticed between the FAE of the jejunal and ileal PP. After an incubation of 1 h, the situation was not changed compared to an incubation of 15 min and no further accumulation of the F4 in the FAE or dome region could be observed.

The binding and endocytosis of the non-labeled F4 to and by the FAE were studied by dual immunohistochemical staining of F4 and cytokeratin 18. No differences were found with the FLUOS-labeled F4 except for the fact the staining intensity was less and therefore endocytosis and translocation were less obvious in loops incubated with the F4 compared to the FLUOS-labeled F4.

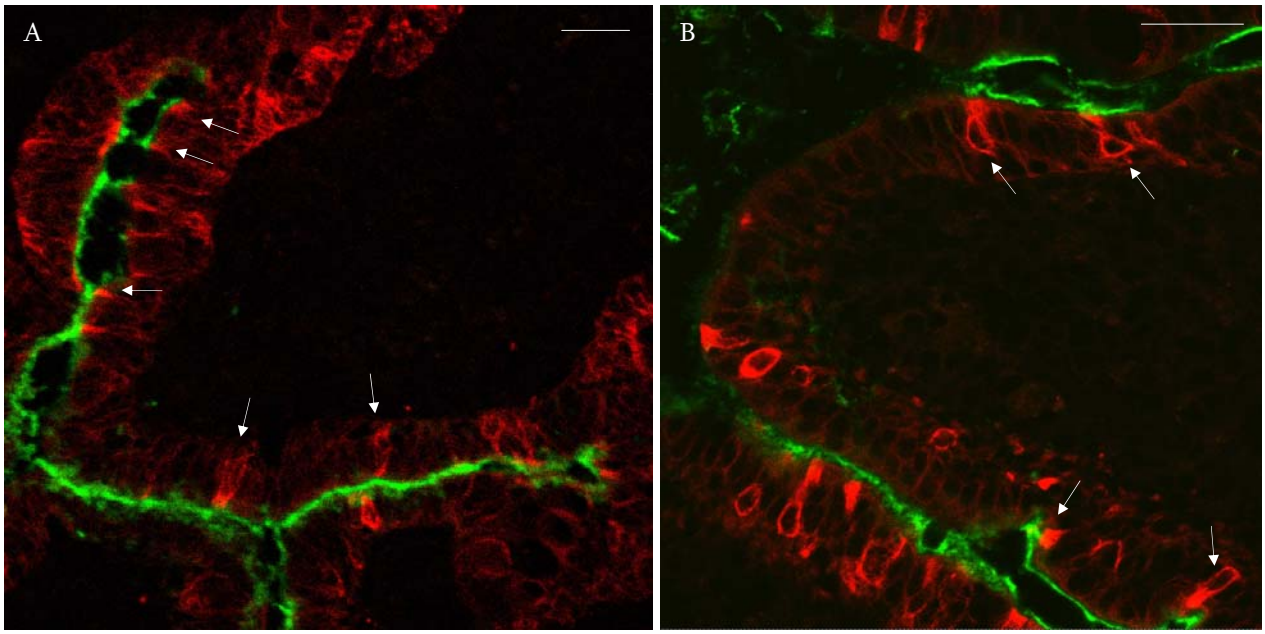

Fig. 6.7. Cryosection of the jejunal or ileal Peyer's patch of an F4R+ pig after 15 min *in vivo* incubation with F4 or FLUOS-labeled F4. Confocal view of the FAE of the jejunal (A) or ileal Peyer's patch (B) after dual immunohistochemical detection of cytokeratin 18 by the specific MAb (*red* fluorescence) and F4 by the F4-specific MAb antibody (A) or FLUOS-labeled F4 (B) (*green* fluorescence). M cells are intensely stained by the cytokeratin 18-specific MAb; a dense plaque is visible in the most apical cytoplasm. F4 clearly have bound to the apical membrane of M cells (*white* arrows). Bars, 36 µm (A), 40 µm (B).

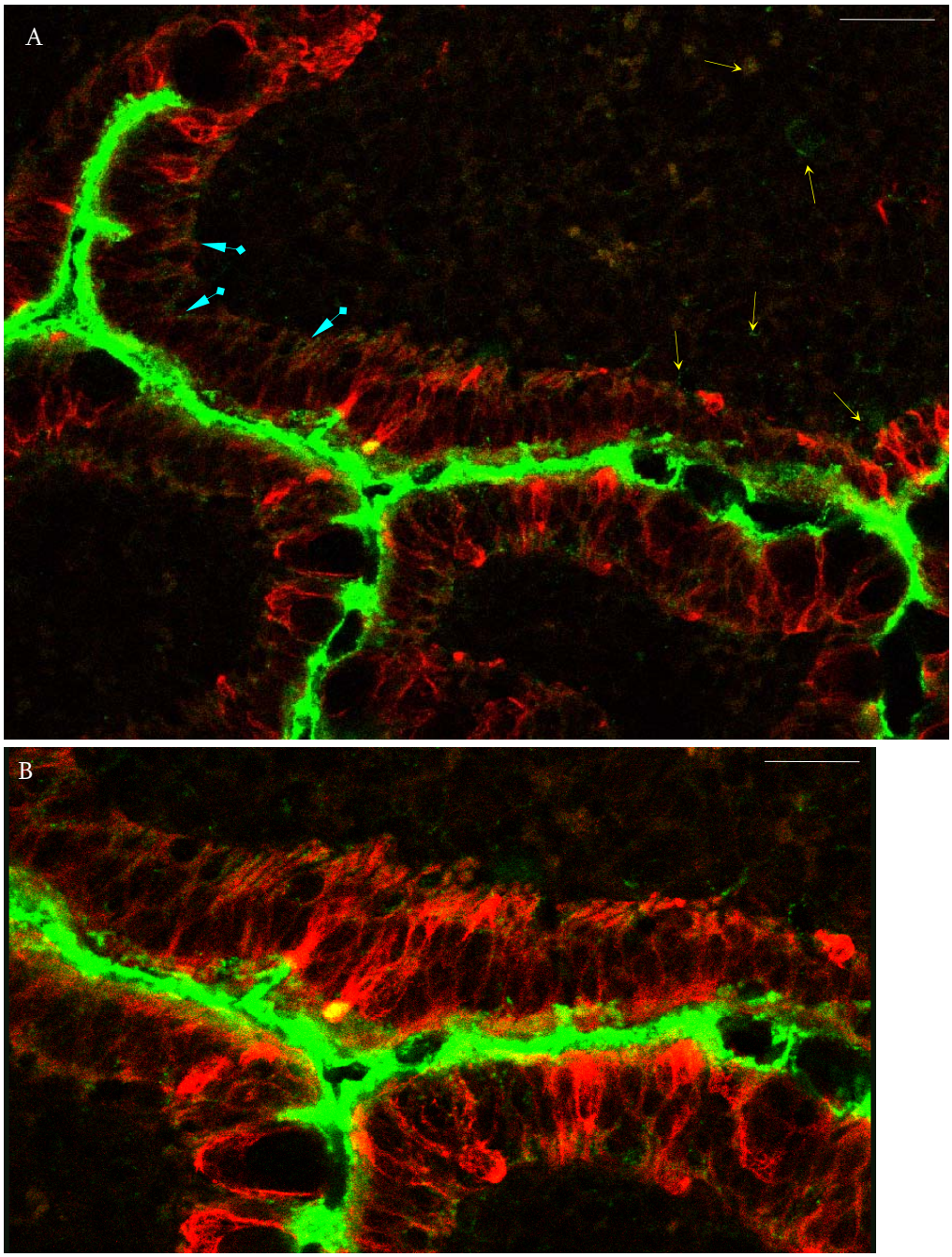

Fig. 6.8. Cryosection of the jejunal Peyer's patch of an F4R+ pig after 15 min *in vivo* incubation with FLUOS-labeled F4. Confocal view of the FAE of the jejunal Peyer's patch after dual immunohistochemical detection of cytokeratin 18 by the specific MAb (*red* fluorescence) and FLUOS-labeled F4 (*green* fluorescence). M cells and goblet cells are intensely stained by the cytokeratin 18-specific MAb. F4 was identified in the most apical cytoplasm of the M cell (*yellow* fluorescence results from *red* and *green* overlapping) and in the follicle-associated enterocytes (*blue* arrows) and further in the dome region of the Peyer's patch (*yellow* arrows). Bars, 50 μm (A), 75 μm (B).

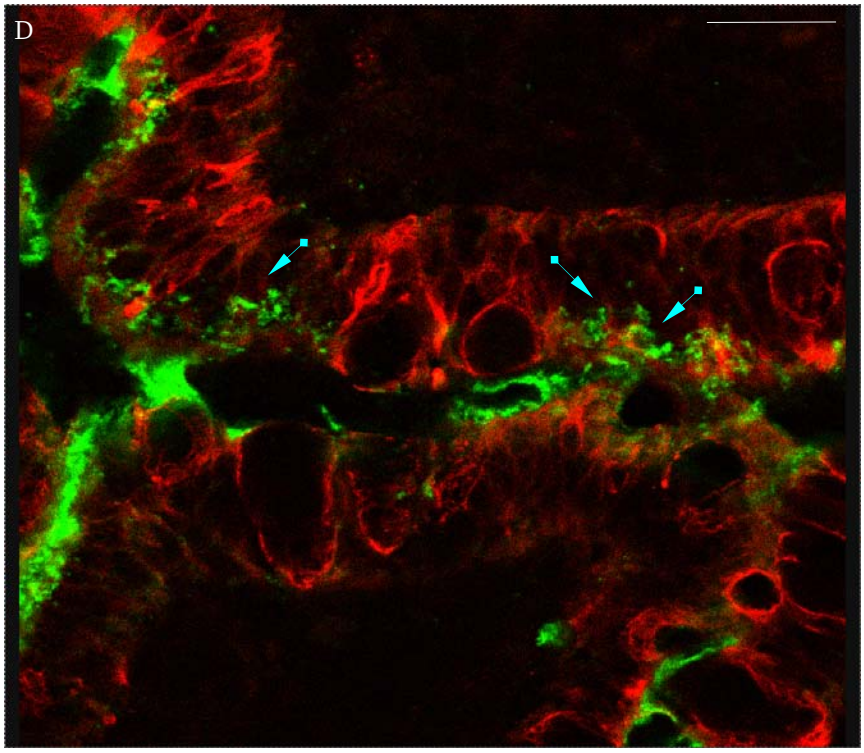

Fig. 6.9. Cryosection of the jejunal Peyer's patch of an F4R+ pig after 15 min *in vivo* incubation with FLUOS-labeled F4. Confocal view of the FAE of the jejunal after dual immunohistochemical detection of cytokeratin 18 by the specific MAb (*red* fluorescence) and FLUOS-labeled F4 (*green* fluorescence). M cells and goblet cells are intensely stained by the cytokeratin 18-specific MAb. F4 was identified in the follicle-associated enterocytes. Bar, 30 µm.

6.5. DISCUSSION

In the present study, F4 and FLUOS-labeled F4 were incubated in jejunal loops with or without a discrete PP and in ileal loops in one F4R- pig and 9 F4R+ pigs. Immunohistochemical analysis of the tissue sections of the different loops demonstrated that, as expected, F4 adhered to the intestinal epithelium of the F4R+ pigs but not of the F4R- pig.

In F4R+ pigs, F4 strongly adhered to the villous epithelium. No difference in F4 adhesion was found between jejunal loops with or without a discrete PP and ileal loops. These findings are consistent with different studies reporting presence of the F4R (Cox and Houvenaghel, 1993; Chandler *et al.*, 1994) and with colonisation of the intestinal mucosa by F4+*E. coli* throughout the small intestine (Hampson *et al.*, 1985; Hinton *et al.*, 1985). Furthermore, F4 bound equally strong to villi at the mesenteric and antimesenteric side. This adhesion was seen at the sides as well as at the tip of the villi, whereas the crypt cells remained negative. Since the intestinal epithelial cells originate from multipotent stem cells present in the crypt, after which they migrate upwards to the villous tip during their differentiation (Cheng and Leblond, 1974; Hermiston *et al.*, 1993), the results suggest that F4R expression by the enterocytes is acquired during their differentiation. However, after a combined infection of pigs with transmissible gastroenteritis virus and F4ac+ETEC, most colonisation occurred at the sides and base of the villi, but seldom at the villous tips (Cox *et al.*, 1988). This suggests that exposure of villi to the flow of the gastrointestinal content can prevent adhesion to villous tips more than to the villous base. In our loop experiments, such washing effects are absent allowing adhesion of F4 to the villous tip.

Besides the adhesion to the villi, F4 also strongly adhered to the FAE overlying the jejunal and ileal PP as up to 100% of the FAE were positive for F4 adhesion. Moreover, F4 adhered to the follicle-associated enterocytes and to M cells, indicating that both cell types can express the F4R. Nevertheless, F4 did not adhere to 5% of the M cells, suggesting that not all M cells express these F4R. This probably reflects different differentiation states of the individual M cells, since the F4R is highly glycosylated (Francis *et al.*, 1999; Jin and Zhao, 2000; Grange *et al.*, 2002) and lectin-binding studies

have revealed that different M cells can exhibit variation in glycosylate expression within the same FAE (Gebert and Posselt, 1997).

No significant differences could be found in F4 adhesion to the villous epithelium and the FAE between incubation periods of 5 min, 15 min and 1 h, indicating that F4 adheres quickly to its receptor. However, after 5 min incubation the staining intensity and the thickness of the F4 band at the brush border was less, demonstrating that the binding to the epithelium becomes stronger with longer incubation periods. Furthermore, variation in F4 adhesion was observed between the individual F4R+ pigs, demonstrating that the F4 adhesion is not as fast or accurate in all F4R+ pigs. This can be due to the presence of F4-specific antibodies in the intestinal lumen, to a weak F4R expression, to the presence of large amounts of intestinal content, or to expression of different F4R. Indeed, Francis *et al.* (1998) and Rutter *et al.* (1975) identified pigs whose brush borders supported the adherence of F4+ *E. coli* but which were resistant to disease caused by those organisms. These piglets did not express IMTGP, an intestinal mucin-type sialoglycoprotein, on their enterocyte membranes (Francis *et al.*, 1998). Francis *et al.* (1998) suggested that besides IMTGP, another F4ac-receptor can be present in the brush borders, allowing adherence of the F4+ *E. coli in vitro* but that both receptors are necessary for bacterial colonisation. Consequently, different degrees of IMTGP production might explain the lower F4 adhesion in some F4R+ pigs.

The present study further demonstrates that following F4R binding, the F4 is endocytosed and translocated across the epithelium by both the villous enterocytes as well as the cells of the FAE. Although F4 could be identified in the apical cytoplasm of M cells, its subsequent transcytosis by these cells could not be demonstrated. Transmission electron microscopy will be necessary to elucidate whether F4 is delivered into the intraepithelial pocket of the M cell following F4R binding. Russell-Jones (2001) suggested receptor-mediated endocytosis of K99 and 987P fimbriae (Russell-Jones, 2001). Binding of these fimbriae to the intestinal villous cell is suggested to result in endocytosis and transcytosis of the fimbriae across the cell and into the circulation, explaining the immune response seen following oral immunisation of mice with these antigens. The endocytosis

and transcytosis of F4 may explain the immunogenicity of these fimbriae upon oral administration, as once F4 is delivered in the LP or the dome region of the PP, it can be taken up by professional antigen presenting cells, which in turn may activate the immune system (Snoeck *et al.*, submitted (a)). However, data about the functional activity of the F4R are lacking. As a consequence, it is not known whether the F4 binding to the F4R itself activates processes, such as the induction of proinflammatory cytokines and chemokines by the epithelial cell (Kagnoff and Eckmann, 1997; Stadnyk, 2002) which may augment the immune response and thus the immunogenicity of the F4. Nevertheless, after release of F4 in the small intestinal LP, F4 could occasionally be identified in SWC3$^+$ cells. The SWC3-specific antibody stains a heterogenous population of cells of myeloid origin present in the porcine small intestinal LP, including mast cells, neutrophils, non-professional antigen presenting MHCII$^+$ eosinophils, and professional antigen presenting MHCII$^+$ macrophages and dendritic cells but not lymphocytes (Stokes *et al.*, 1996; Haverson *et al.*, 2000; Summerfield *et al.*, 2003). Furthermore, F4 could rarely be found in SWC3$^-$ cells. Based on the cell morphology, these SWC3$^-$ cells do not represent B cells, but probably plasmacytoid DCs which do not always express detectable levels of SWC3 (Riffault *et al.*, 1977, 2001). So, some SWC3$^+$ and SWC3$^-$ cells which have taken up F4 in the LP may present the F4 to T cells in the mesenteric lymph nodes, resulting in an immune response. Indeed, we have previously demonstrated that following targeting of the F4 to the LP an F4-specific antibody response is induced (Snoeck *et al.*, submitted (a)). However, further characterisation of these SWC3$^+$ cells was not performed.

We have previously demonstrated that an intestinal F4-specific antibody response is better induced following targeting of the F4 fimbriae to the jejunal PP than following targeting to the LP (Snoeck *et al.*, submitted (a)). Since intestinal F4 can be delivered to the dome region of the PP via translocation through the FAE as demonstrated in this study, the immune response induced in these organised lymphoid tissues is probably more important than this in the LP. However, the ileal PP seem to be less important in the induction of the intestinal F4-specific antibody response as compared to the jejunal PP (Snoeck *et al.*, submitted (a)). This can not be explained by a lesser F4 binding to the ileal

FAE as compared to the jejunal FAE, since no difference could be found between these 2 types of PP. Nevertheless, using intestinal loops injected with yeast cells, Gebert *et al.* (1994) demonstrated that the number of yeast cells taken up in the jejunal PP far exceeded that in the ileal PP which they attributed to the functional differences observed between these two types of PP (Binns and Licence, 1985). Differences in uptake of F4 between the FAE of the jejunal and ileal PP can not be excluded in the present study since no quantification of F4 uptake was performed.

The endocytosis and transcytosis of F4 could be detected after 15 min, but not as early as after 5 min incubation. Furthermore, after 1 h incubation the situation remained the same as after 15 min incubation and no further accumulation of the F4 in the LP or dome region could be found. Moreover, F4 could never be detected in the B cell follicles or interfollicular regions of the PP. This may suggest that once the F4 is taken up by immune cells, including professional antigen presenting cells, the F4 is degraded causing loss of fluorescence of the FLUOS-labeled F4 and epitopes for the F4-specific MAb binding or that an incubation period of 1 h is too short to identify F4 in these parts of the PP. Moreover, the endocytosis and translocation of F4 was more obvious when FLUOS-labeled F4 was used, suggesting a partial degradation of the F4 during translocation resulting in less F4-specific MAb binding. On the other hand, the transcytosis of the FLUOS-labeled F4 demonstrates that F4 can be used as carrier for the translocation through the epithelial barrier of other small molecules and may open new perspectives for oral vaccine development using F4 as carrier for other antigens. Furthermore, this may suggest that the F4 can be used to co-transport larger molecules. However further study is necessary to confirm this. Russell-Jones (2001) suggested that K99 and 987P fimbriae can co-transport BSA from the intestine into the circulation following receptor-mediated endocytosis and transcytosis after oral administration of BSA-fimbriae conjugates. However binding of these fimbriae to enterocytes was not shown and receptor-mediated endocytosis was not confirmed by immunohistochemical analysis. Furthermore, there are no studies showing the presence of K99R or 987PR on mouse small intestinal villi and K99+ or 987P+ ETEC are not pathogenic for mice, as a consequence it is not clear whether

in the study of Russell-Jones (2001) rather uptake by M cells than by enterocytes is responsible for the observed immune response following oral administration of BSA-fimbriae conjugates.

In the present study only one F4R- pig was tested for adhesion to their villi. However, no adhesion was observed in 6 different loops after 2 different incubation periods. Furthermore, the absence of F4 adhesion to the intestinal epithelium in the F4R- pig is in agreement with the absence of F4-specific ASC induction following oral F4 administration and challenge with F4+*E. coli* in F4R- pigs (Van den Broeck *et al.*, 1999a) and suggests that F4 is not able to penetrate through the epithelial layer and to reach the cells of the underlying immune system in sufficient amounts to induce ASC. This supports the hypothesis that the F4R on the intestinal epithelium plays an important role in the induction of the mucosal immune response following oral F4 administration.

6.6. CONCLUSION

This is the first study that demonstrates that fimbriae, namely F4 fimbriae, can be endocytosed in and translocated across the intestinal epithelium in F4R+ pigs. The F4R not only concentrate the F4 on the intestinal epithelium but are also of importance for its subsequent endocytosis and transcytosis. This occurs both by the villous enterocytes as well as by the cells of the FAE above the PP. Once the epithelial layer is crossed, the F4 can activate the gut-associated immune system in the Peyer's patches and the LP. Furthermore, evidence is provided that F4 probably can be used as carrier for the translocation of other small molecules across the intestinal epithelium and opens new perspective for vaccine development using F4 as carrier for other antigens.

6.7. ACKNOWLEDGEMENTS

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CHAPTER 7

ENTERIC-COATED PELLETS OF F4 FIMBRIAE FOR ORAL VACCINATION OF SUCKLING PIGLETS AGAINST ENTEROTOXIGENIC ESCHERICHIA COLI INFECTIONS

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7.1. ABSTRACT

To prevent enterotoxigenic *Escherichia coli* (ETEC) induced postweaning diarrhoea, the piglet needs an active mucosal immunity at the moment of weaning. In the present study, the feasibility of oral vaccination of suckling piglets against F4$^+$ETEC infection with F4 (K88) fimbriae was studied. Furthermore, oral vaccination with enteric-coated pellets of F4 fimbriae was compared to vaccination with F4 fimbriae in solution. Therefore, piglets were orally administered 1 mg F4 fimbriae in pellets or in solution during 3 successive days at the age of 7 and 21 days, whereas control piglets were not vaccinated. Five days postweaning (33 days of age), all animals were orally challenged with F4$^+$ETEC. Despite the induction of an immune response upon oral administration of both F4 fimbriae in pellets as in solution, the colonisation of the small intestine by F4$^+$ETEC upon oral challenge could not be prevented. However a marginal but significant reduction in F4$^+$*E. coli* faecal excretion was found in the piglets vaccinated with F4 fimbriae in pellets, indicating that the use of an enteric-coat which protects the F4 fimbriae against inactivation by milk factors and degradation by enzymes improves vaccination.

7.2. INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) are an important cause of diarrhoea and mortality in neonatal (Alexander, 1994) and recently weaned (Hampson, 1994) piglets. Neonatal infections can be effectively prevented by passive colostral and lactogenic immunity which can be obtained and increased by vaccination of the sow (Rutter and Jones, 1973; Deprez *et al.*, 1986). Vaccination of piglets against postweaning infections is still an important challenge. Since newly weaned piglets are deprived of passive lactogenic immunity, active immunisation is needed for protection. An efficient activation of the protective intestinal mucosal immune mechanisms can occur following oral infection but is not obtained by parenteral immunisation, which tends to stimulate the systemic rather than the mucosal immune system (Moon and Bunn, 1993). Moreover, competent oral vaccines for inducing mucosal protection are not yet available.

Some of the ETEC strains bear F4 fimbriae which allow these bacteria to adhere to F4-specific receptors (F4R) present on brush borders of villous enterocytes. Consequently,

colonisation of the small intestine can occur. The presence or absence of these F4R is based on genetic inheritance, with F4R expression being the dominant character (Gibbons *et al.*, 1977). The F4 mediated adhesion is a prerequisite for infection, as piglets without the F4R are resistant to F4$^+$ETEC infections (Rutter *et al.*, 1975; Gibbons *et al.*, 1977). It has been demonstrated that newly weaned F4R positive piglets can be orally immunised with detached F4 fimbriae in solution (Van den Broeck *et al.*, 1999a). However, to prevent postweaning diarrhoea, an active mucosal immunity is needed at the moment of weaning. Therefore the piglets have to be immunised during the suckling period. Oral solutions are unpractical for regular administration to suckling animals. A vaccine formulation which can be mixed with creep feed will be more appropriate. For this purpose enteric-coated pellets were made, since enteric-coated formulations have been efficiently used for oral administration of vaccines (O'Hagan, 1998; Singh and O'Hagan, 1998). They have the important advantage that the enteric coating protects the antigen against possible detrimental effects of acids, enzymes etc. present in the stomach and the duodenum (Snoeck *et al.*, 2004b).

At this moment studies report vaccination of piglets against postweaning diarrhoea during the suckling period. In the present study, we examined the feasibility of oral vaccination of suckling piglets with F4 fimbriae against F4$^+$ETEC infection. Furthermore, we compared oral vaccination with enteric-coated pellets of F4 fimbriae to those with F4 fimbriae in solution. The pellets do not disintegrate at a pH equal to or below 4.8, the highest pH measured in the stomach of suckling piglets, while disintegration occurs within 10 min after exposure to a phosphate buffer (37°C) with a pH of 6.3 (Huyghebaert, Snoeck (equally contributed) *et al.*, submitted), the lowest pH measured at the target site (the beginning of the jejunum) of suckling piglets (Snoeck *et al.*, 2004b). The immune response upon oral vaccination was examined by determining the F4-specific IgG, IgA and IgM serum antibodies, and protection was evaluated by oral challenge with F4$^+$ETEC.

7.3. MATERIAL AND METHODS

7.3.1. Experimental animals

Fourteen suckling piglets (3 litters, Belgian Landrace x English Landrace), seronegative for antibodies against F4, were housed in a conventional farm in the breeding house at 30 °C ± 3 °C together with the sows, which were also F4-antibody-negative in serum and milk as determined by ELISA. At the age of 28 days, the piglets were weaned and were brought to the Faculty where they were housed together in 1 isolation unit with IR-lamps for local heating at 24 ± 2 °C with food and water ad libitum. To prevent outbreaks of *E. coli* infections, all animals were orally treated during 3 successive days with colistine (150, 000 U/kg body weight; Colivet, Prodivet Pharmaceuticals, Eynatten, Belgium) from 1 day before weaning. Experimental procedures and animal management procedures were undertaken in accordance with the requirements of the animal care and ethics committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2003).

7.3.2. Bacterial inoculum

The *E. coli* strain GIS 26 (O149:K91:F4ac, LT^+, STa^+, STb^+), was cultured for 18 h in Tryptone Soya Broth (Oxoid, Basingstoke, England) at 37°C and 85 rpm. Bacteria were collected by centrifugation (2000 x g; 35 min, 4°C) and washed twice with phosphate-buffered saline (PBS; 150 mM, pH 7.4). Subsequently, the bacteria were suspended in PBS to a final concentration of 10^9 bacteria/ml as determined by measuring the optical density at 660 nm (OD_{660}). An OD of 1 equals 10^9 bacteria/ml, as determined by counting the colony forming units.

7.3.3. Purification of F4 fimbriae

The F4 fimbriae of the bacteria were isolated as previously described (Van den Broeck et al., 1999c). The protein concentration of the isolated fimbrial solution was determined by the bicinchoninic acid reaction (Sigma-Aldrich, Bornem, Belgium) with bovine serum albumin as standard.

7.3.4. Preparation of enteric-coated F4 pellets

7.3.4.1. Production of pellets

Microcrystalline cellulose containing 11.3-18.8% (g/g) sodium carboxymethyl cellulose (Avicel ®CL 611, FMC Europe, Brussels, Belgium) and α-lactose MH (200 Mesh, Pharmatose®, De Melkindustrie, Veghel, the Netherlands) were used as excipients for the production of pellets. Sodium carboxymethyl starch (Explotab®, Penwest Pharmaceuticals, NY, US) was used as a disintegrant. The F4 fimbrial solution was used as granulation fluid. A mixture consisting of 2.5% Avicel ®CL 611, 10% Explotab® and 87.5% α-lactose MH 200 Mesh was preblended and granulated with the F4-solution (70% v/w), so that 4 mg of the isolated F4 fimbriae were incorporated in 1 g pellets. Extrusion was performed in a single screw extruder (Dome extruder lab model DG-L1, Fuji Paudal Co., Tokyo, Japan) at 45 rpm, through a 1 mm perforated screen. The extrudate was spheronized on a mini-spheronizer (Caleva, Sturminster Newton, UK) using a cross-hatched friction plate, operating at 1680 rpm with a residence time of 2 min. The wet spheres were dried in a fluid bed dryer (GPCG-1, Glatt, Binzen, Germany) with an inlet air temperature of 25 °C.

7.3.4.2. Enteric coating of the pellets

The pellets were coated with Eudragit®L 30 D-55. To prepare the coating dispersions, a 30% (w/w) aqueous Eudragit® dispersion was used (Röhm, Darmstadt, Germany). Excipients used for the coating dispersions were triethyl citrate (TEC; 20% on dry polymer; Sigma-Aldrich, Bornem, Belgium) as a plasticizer, polysorbate 80 (33% aqueous solution; 10% on dry polymer; Tween® 80, Alpha Pharma, Braine-l'Alleud, Belgium) as an emulsifier and glyceryl monostearate (GMS; 8% on dry polymer; Federa, Braine-l'Alleud, Belgium) as a glidant. The solid and the polymer content of the coating dispersion were 20% (w/w) and 15.27% (w/w), respectively. The coating dispersion was prepared by adding TEC, GMS and Tween® 80 to water under stirring over a period of 10 min with a high-speed mixer (Silverson, Bucks, England). The dispersion was gently added to the Eudragit® dispersion and homogenized by magnetic stirring. For the coating experiments, 300 g pellets were coated in a fluid bed coating apparatus (GPCG-1, Glatt,

Binzen, Germany), used in the bottom spray mode with the Würster setup (nozzle diameter 0.8 mm, spraying rate 3.5-4 g/min, atomizing pressure 1.5 bar, outlet air temperature 23-25 °C). The pellets were coated with 15% (w/w) Eudragit® L 30 D-55. After coating, the pellets were cured for 15 min at the same conditions followed by 24 h on silica at 8 °C before administration to the piglets.

7.3.4.3. Determination of the concentration F4 fimbriae in pellets

To determine the concentration of the F4 fimbriae in the pellets, the F4 fimbriae were extracted from the pellets. Ten ml PBS (pH 7.4) was added to 1 g pellets followed by gently shaken this suspension during 1 h at room temperature. The suspension was filtered through a Whatman filter 50 (VWR Internations, Leuven, Belgium). The concentration F4 fimbriae in the filtered solution was determined by indirect ELISA, using the F4 fimbrial solution as standard (Huyghebaert, Snoeck (equally contribution) *et al.*, submitted).

7.3.5. Experimental procedure

At the age of 7 days, 3 animals were given orally via a syringe 1 mg F4 fimbriae in 5 ml PBS (soluble group) on 3 successive days. Five animals received a similar immunisation with the F4 fimbriae (1 mg) in enteric-coated pellets (pellet group). The pellets were orally administered with 5 ml PBS supplemented with citric acid to a pH of 5 to prevent dissolution of the enteric coating. The direct oral administration of a suspension of pellets allowed to give the same dose F4 as in the soluble group (which would have been impossible if pellets were mixed with the creep feed). Another 6 animals were not immunised (control group). Each immunisation procedure (F4 fimbriae in solution or pellets or none) was restricted to 1 litter, considering the risk of contact of the control animals with the F4 antigen after oral immunisation (unpublished observation). Both vaccinated groups received identical booster immunisations during 3 consecutive days at the age of 21 days. Five days postweaning, at the age of 33 days, all animals were orally infected with the virulent $F4^+$ETEC strain as described previously (Cox et al., 1991). Briefly, piglets were treated with florphenicol in PBS (20 mg/kg/day) (Nuflor, Schering-Phough NV, Brussels, Belgium), at days 3 and 2 pre-infection. They were fasted overnight and were deprived of water 3 h pre-infection. Subsequently, they were orally infected

with 10^{10} F4$^+$ETEC, 15 to 30 min after neutralising the acidic gastric pH with 60 ml of $NaHCO_3$ (1.4 % (w/v) in distilled water). At the age of 47 days, the animals were killed and jejunal villi were isolated to determine the presence of F4R on the villous enterocytes. Only the F4R positive animals were included in the results.

7.3.6. Faecal excretion of F4$^+$ETEC

Faecal samples were collected daily from the day of the challenge until 7 days post challenge to determine the faecal F4$^+$ *E. coli* excretion. Samples were analysed immediately as storage is harmful to the bacteria. One percent (w/v) suspensions were made in PBS at 4 °C. Excretion of ETEC in faeces was demonstrated by inoculating 50 µl of diluted faecal samples (10-fold dilutions in PBS from 1% to 1 x 10^{-5} %) onto blood agar plates (Difco Laboratories, Detroit, USA) at 37°C for 24 h and was quantified using dot blotting as described by Van den Broeck *et al.* (1999a).

7.3.7. ELISA for F4-specific IgG, IgA and IgM.

Blood was sampled from the jugular vein at 7, 14, 21, 33, 40 and 47 days of age for determining the F4-specific IgG, IgM and IgA titers in serum. Serum was collected and inactivated at 56 °C and subsequently treated with kaolin (Sigma-Aldrich, Bornem, Belgium) to decrease the background reading in the ELISA as described previously (Van den Broeck et al., 1999a). The serum was diluted in ELISA dilution buffer [PBS containing 0.05% (v/v) Tween® 20 (Merck, Hohenbrunn, Germany) and 3% (w/v) bovine serum albumin (Sigma-Aldrich, Bornem, Belgium)] to obtain a final serum dilution of 1/10. F4-specific antibodies were detected using the indirect ELISA described by Van den Broeck *et al.* (1999a), except for the anti-swine IgG-, IgM-, and IgA-conjugates. These conjugates had been prepared by coupling the swine IgG-, IgM- and IgA-specific Mab (Van Zaane and Hulst, 1987) to biotin using the biotin labeling kit (Roche Diagnostics, Mannheim, Germany). The incubation (1h 37°C) of the biotinylated conjugates was followed by addition of HRP-conjugated streptavidin (GIBCO BRL, Merelbeke, Belgium) to the wells for 1 h at 37°C. The obtained ODs of all the sera (dilutions, 1/10) at the age of 7 days were averaged, and the standard deviation was calculated. The mean, increased by twice the standard deviation, was considered as cutoff value. The cutoff values were 0.392, 0.450,

0.490 for F4-specific IgM, IgA and IgG, respectively. The antibody titer was the inverse of the highest dilution which still had an OD_{405} higher than the calculated cutoff value.

7.3.8. *In vitro* villous adhesion assay for F4R characterisation

In order to determine the presence of F4R on the small intestinal villous enterocytes, an *in vitro* villous-adhesion assay was performed at the end of the experiment at the age of 47 days as described previously (Van den Broeck *et al.*, 1999a). Adhesion of less than 5, less than 30 and more than 30 bacteria per 250 µm villous brush border length was noted as negative, weak or strong positive, respectively (Cox and Houvenaghel, 1993).

7.3.9. *In vitro* inhibition villous adhesion assay with F4 fimbriae and milk serum

In order to determine the influence of sow's milk on the oral immunisation with F4 fimbriae, an *in vitro* inhibition villous adhesion assay, based on the test of Girardeau (1980), was performed in the presence of milk serum without F4 specific antibodies. Briefly, prior to the addition of $F4^+$ETEC, the villi were pre-incubated with 50 µg/ml F4-fimbriae during 1 h at room temperature in the absence or presence of 20% (v/v) and 50% (v/v) milk serum in PBS with 1% D-mannose. Milk was collected and milk serum was prepared as previously described (Callebaut *et al.*, 1990).

7.3.10. Satistical analysis

Statistical analysis was performed using the software package SPSS version 11.0. Differences in $\log_2$ antibody serum titers (IgM, IgA, IgG) and in $\log_{10}$ faecal ETEC excretions between the groups and between different time points within the groups were tested for statistical significance using General Linear Model using the following syntax:

GLM; Time1 time 2 time 3….time x BY group; /WSFACTOR = time x SIMPLE; /METHOD = SSTYPE (3); /CRITERIA = ALPHA (.05); /EMMEANS = TABLES (group*time) COMPARE (group); /EMMEANS = TABLES (group*time) COMPARE (time); /WSDESIGN = time; /DESIGN = group

Differences in duration of faecal *$F4^+$E. coli* excretion between the groups was tested using one way ANOVA. The significance level was set at 5%. P-values between 0.05 and 0.1 were mentioned to describe a trend rather than a significant difference and were indicated by $P<0.1$.

7.4. RESULTS

7.4.1. F4R characterisation of the experimental animals

The *in vitro* villous adhesion assay revealed that 10 animals were strong F4R positive, 3 were F4R negative (2 piglets of the control and 1 of the pellet group) and 1 was weak F4R positive (control group). The latter four animals remained F4-seronegative throughout the experiment and excreted much less $F4^+$ *E. coli* upon challenge (only excretion during the first 3 days post challenge, most likely due to recurrent oral uptake of the bacteria by sustained faecal excretion of $F4^+$ *E. coli* by the F4R positive animals).These animals were therefore excluded from the study. As a consequence, the oral group contained 3 animals, the pellet group 4 and the control group 3.

7.4.2. Serum antibody response after oral vaccination and oral challenge with $F4^+$ETEC

The soluble as well as the pellet group had been orally vaccinated at the age of 7, 8 and 9 days and again at the age of 21, 22 and 23 days. The control group had not been immunised. Five days postweaning (33 days of age), all animals were inocculated with the virulent $F4^+$ETEC strain. The F4-specific serum antibody response was analysed till 47 days of age (Fig. 7.1). Following primary vaccination, a F4-specific antibody response could only be detected in the oral group. At the age of 21 days, the F4-specific IgG, IgA and IgM titers were increased in this group in comparison with the control ($P<0.05$) and the pellet group ($P<0.05$, except for IgA = $P<0.1$). After the second vaccination, the F4-specific IgG ($P<0.1$) and IgA ($P<0.05$) titers were increased in both vaccinated groups. At the age of 33 days, these titers were higher than in the control group ($P<0.05$, except for IgG of pellet group = $P<0.1$), with higher F4-specific IgA levels in the soluble than in the pellet group ($P<0.05$).

After challenge, only the control group displayed an antibody response, with an increase of F4-specific IgG, IgA and IgM titers ($P<0.05$). The F4-specific IgM titers peaked at the age of 40 days, after which they subsided ($P<0.05$). Contrary, the F4-specific IgG titer increased till 47 days of age ($P<0.05$), whereas the F4-specific IgA remained at the same level. The challenge infection of the pellet and the soluble group evoked no serum antibody booster response. The F4-specific IgG titer in the soluble group did not change,

whereas in the pellet group it was decreased at 47 days of age ($P<0.05$). In both the soluble ($P<0.05$) and the pellet group ($P<0.1$) the IgA decreased, whereas the F4-specific IgM remained at background levels, indicating absence of restimulation and consequently protection.

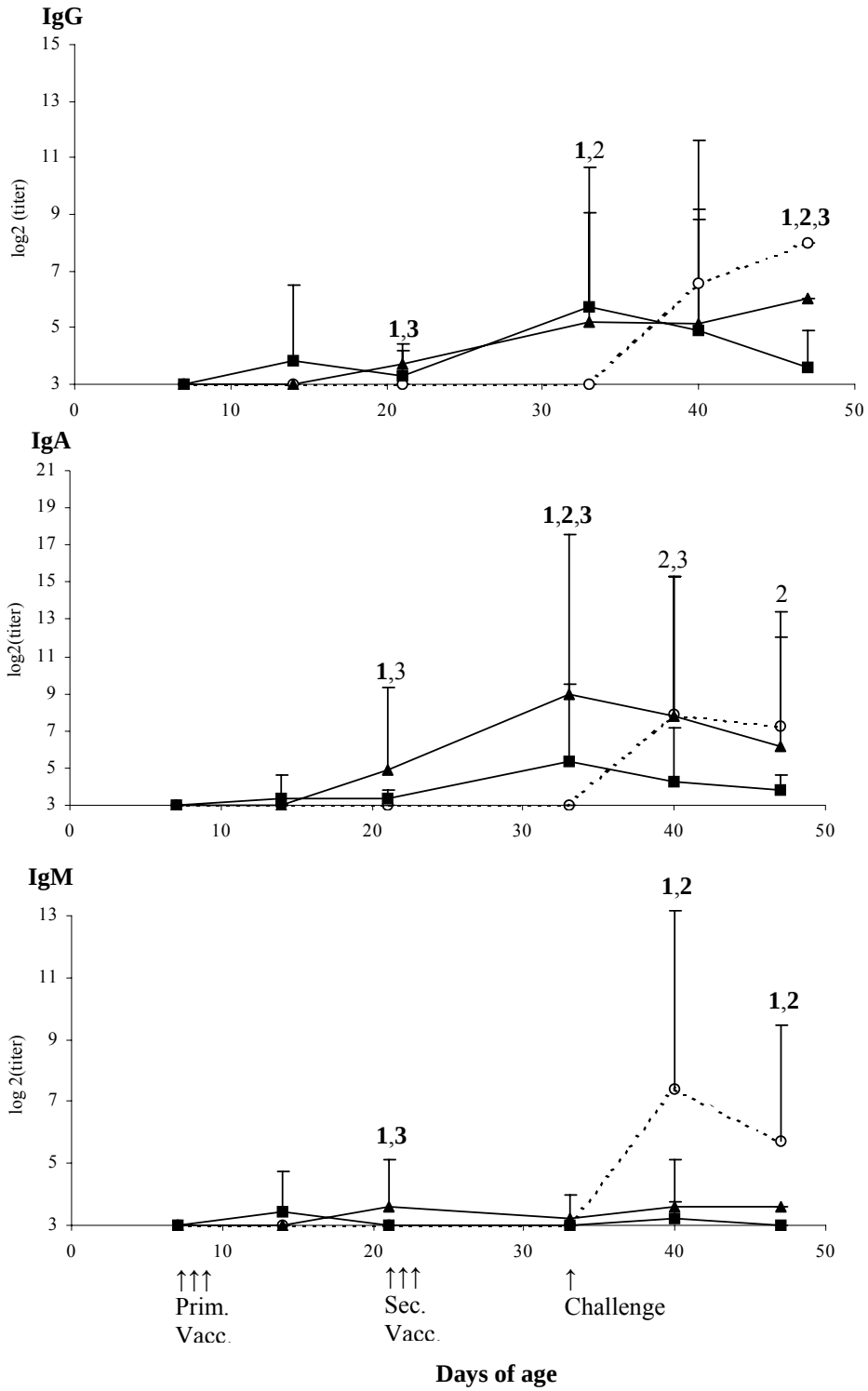

Fig. 7.1. Kinetics of the F4-specific IgG, IgA and IgM serum antibody titers (± SEM) in the control group (- - ○- -, n = 3), the pellet group (■, n = 4) and the soluble group (▲, n = 3). Vaccination and challenge procedure are given in the material and methods section. Significant differences between the control group and the soluble group were indicated with [1], between the control group and the pellet group with [2], and between the soluble group and the pellet group with [3]. A bold number indicates $P<0.05$, otherwise $P<0.1$ Significant differences between the different time points are mentioned in the text.

7.4.3. F4+*E. coli* excretion after challenge with F4+ETEC

An examination of the faecal samples revealed that all groups excreted hemolytic F4+ETEC after challenge (Fig. 7.2). However, from 2 days post challenge (dpc) onwards (day 2, $P < 0.1$; day 3 to 5, $P < 0.05$), the excretion was significantly reduced in the pellet group. Moreover, the duration of the faecal F4+*E. coli* excretion was significantly reduced in the pellet and not in the soluble group ($P < 0.1$), no excretion was found in the pellet group 6 dpc in contrast to the soluble and the control group.

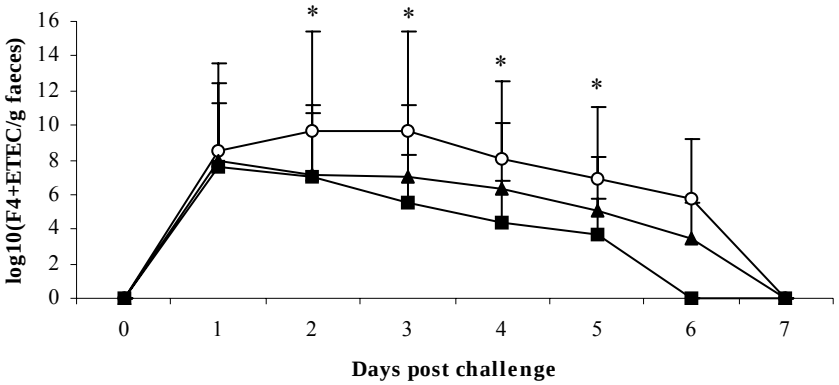

Fig. 7.2. Faecal excretion of hemolytic F4+ETEC/g faeces (log10 (mean number/g faeces) ± SEM) after oral challenge with F4+ ETEC of the control (- -○- -, n = 3), the pellet (■, n = 4), and the soluble group (▲, n = 3). Significant differences between the control group and the pellet group were indicated with * (day 2, $P<0.1$; day 3 to 5, $P<0.05$).

7.4.4. Inhibition villous adhesion assay with F4 fimbriae and milk serum using F4+ETEC

Since the piglets were vaccinated during the suckling period, milk could interfere with the immunisation by inhibiting binding of F4 to its receptor. In the *in vitro* inhibition villous adhesion assay, it was shown that the reduction in adhesion of F4+ETEC to the villus, obtained by pre-incubating the villi with 50 µg/ml F4 fimbriae, was completely abolished by adding 20 % and 50 % milk serum without F4-specific antibodies to the F4-fimbriae during the pre-incubation (Fig. 7.3).

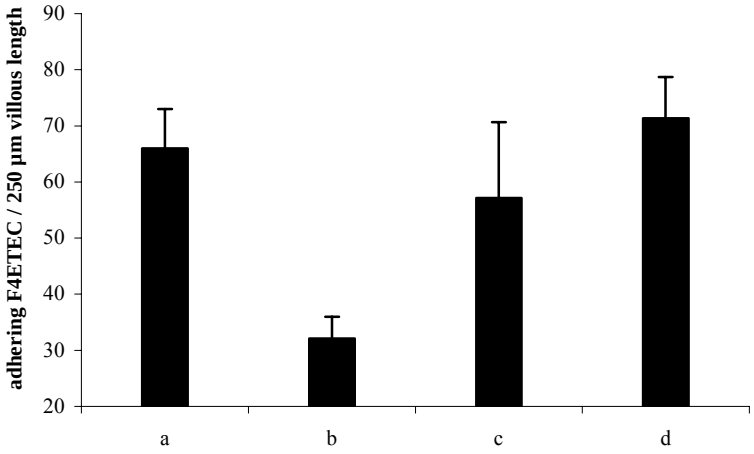

Fig. 7.3. Number of adhering F4+ETEC per 250 µm length of intestinal villous brush border (n = 3, ± SD) without pre-incubation (a) and with pre-incubation with 50 µg/ml F4 fimbriae (b), 50 µg/ml F4 fimbriae and 20% (c) or 50% milk without F4 specific antibodies (d).

7.5. DISCUSSION

In the present study, we examined the feasibility of oral vaccination of suckling piglets with F4 fimbriae against F4$^+$ETEC infection. Furthermore, we compared oral vaccination with enteric-coated pellets of F4 fimbriae to this with F4 fimbriae in solution. Characterisation of the F4R status of the piglets demonstrated that a high percentage of the animals were F4R negative, even though the F4R expression is dominant (Gibbons *et al.*, 1977). Most likely this was due to the fact that we looked for F4-seronegative animals, increasing the chance on finding F4R negative animals. In Belgium, only 35% of the non-vaccinating farms (68% of all farms) are F4-seronegative, indicating a high prevalence of the receptor (Van den Broeck *et al.*, 1999d). There are no indications that there is a selective pressure towards F4R negative animals.

The results of the immunisation experiment provide evidence that F4 fimbriae in solution and in pellets were able to induce an immune response upon oral administration to suckling piglets, with the serum antibody response in the soluble group being significantly higher than in the pellet group. However, in contrast to newly weaned piglets (Van den Broeck *et al.*, 1999a), the induced immune response could not completely prevent colonisation of the small intestine by F4$^+$ETEC upon oral challenge as both the control as well as the vaccinated piglets excreted hemolytic F4$^+$*E. coli* in their faeces for at least 5 days. This could be attributed to the still young mucosal immune system of the suckling piglet (Roth, 1999; Pescovitz, 1998). However, a significant reduction in the F4$^+$*E. coli* excretion could be found in piglets vaccinated with F4 fimbriae in pellets. This indicates that the use of an enteric coat which protects the antigen against degradation and inactivation by enzymes in the stomach and the beginning of the small intestine has beneficial effects. Probably, the F4 fimbriae in solution are partially destroyed by enzymes, by which the amount of antigen, reaching the mucosal inductive sites, is reduced. Furthermore, since milk in the absence of F4 specific antibodies inhibits, the adhesion of F4 fimbriae to the villous brush borders, an enteric coat protects the F4 fimbriae against neutralisation by the milk, allowing a higher efficiency of the vaccination due to a higher antigen load in the beginning of the jejunum. However, in spite of the

better protection upon oral challenge, the serum antibody response was significantly lower in comparison with the piglets vaccinated with F4 fimbriae in solution. In a recent experiment, it was seen that the F4-specific serum antibody response after oral vaccination with F4 fimbriae in solution, is not only the result of an immune response in the small intestine, but also of an induced response in the mouth or pharynx region (Snoeck *et al.*, submitted (a)). This could explain the discrepancy between the induced serum antibody response and the protection against the F4$^+$ETEC infection in the soluble group.

7.6. CONCLUSION

These results clearly demonstrate that oral vaccination of suckling piglets with F4 fimbriae, as done in the present study, can not prevent F4$^+$ETEC excretion upon challenge postweaning. Furthermore, this study indicate that enteric-coated pellets of F4 fimbriae can be used as solid formulation, mixable with creep feed, since oral vaccination with the pellets was beneficial compared to F4 fimbriae in solution. However, to develop a vaccine which can prevent postweaning diarrhoea, further optimisations are required. Increasing the antigen dose and addition of mucosal adjuvants might improve the stimulation of the still young mucosal immune system of the suckling piglet.

7.7. ACKNOWLEDGEMENTS

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PART IV

GENERAL DISCUSSION

CHAPTER 8

GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

In the present study, different parameters necessary for optimal oral vaccine development for piglets were studied. As model system, F4 fimbriae (K88) of enterotoxigenic *Escherichia coli* were used as oral vaccine against F4$^+$ETEC induced postweaning diarrhoea. These F4 fimbriae are proteinaceous surface antigens which play an important role in the pathogenesis as they allow the bacteria to adhere to F4-specific receptors on the villous brush border of the enterocytes resulting in colonisation of the small intestine (Jones and Rutter, 1972). In our laboratory, it has been demonstrated that newly weaned piglets can be orally vaccinated with these F4 fimbriae against an F4$^+$ETEC infection (Van den Broeck *et al.*, 1999a). However, protecting the F4 fimbriae from degradation by gastrointestinal acid and enzymes and delivering them to the major inductive sites of the intestinal mucosal immune system by oral delivery systems would most likely improve the efficacy of the vaccine. Development of oral systems delivering the F4 fimbriae to the inductive sites requires information on the gastrointestinal transit times, which were determined in young pigs *(chapter 3)*. It was further investigated to what extent the F4 fimbriae are degraded by gastric enzymes and intestinal pH *(chapter 4)*. Moreover, the major inductive sites of the F4-specific intestinal immune response were determined *(chapter 5)* as were the cell type(s) responsible for the translocation of the F4 fimbriae across the intestinal epithelial barrier *(chapter 6)*. Finally, in order to evaluate the protective immune response, a challenge experiment was performed with enteric-coated pellets of F4 fimbriae as oral vaccine for suckling piglets and F4$^+$ETEC as challenge strain *(chapter 7)*.

8.1. INDUCTION OF THE F4-SPECIFIC INTESTINAL MUCOSAL IMMUNE RESPONSE FOLLOWING IMMUNISATION OF F4R^{+} PIGS WITH F4 FIMBRIAE

8.1.1. The intestinal inductive sites: the jejunal versus ileal Peyer's patches and lamina propria

The F4 fimbriae were locally injected in the lumen at different sites along the gastrointestinal tract and were selectively targeted to the lamina propria or Peyer's patches by local injection *(chapter 5)*. Analysis of the F4-specific antibody secreting cells (ASC) demonstrated that the highest intestinal immune response was induced following administration of the F4 fimbriae at the vicinity of the jejunal Peyer's patches, with the appearance of ASC mainly in the jejunal and ileal Peyer's patches and mesenteric lymph nodes, and lower amounts in the lamina propria. This indicates that in comparison to the ileal Peyer's patches and the lamina propria, the jejunal Peyer's patches are the major inductive sites of the intestinal immune system. This seems to be consistent with the reports that the 25 to 35 jejunal Peyer's patches, which are distributed along the jejunum and proximal ileum are secondary lymphoid organs, whereas the continuous ileal Peyer's patches may function as a primary lymphoid organ. Indeed, in comparison with the jejunal Peyer's patches, the ileal Peyer's patches have less lymphocyte migration, smaller interfollicular areas and a higher incidence of B cells, which are all characteristics of a primary lymphoid organ (Binns and Licence, 1985; Barman *et al.*, 1997). Furthermore, Andersen *et al.* (1999) demonstrated that the majority (> 90%) of ileal Peyer's patch follicular cells are apoptosis-sensitive immature B cells, suggesting that the ileal Peyer's patch, like the sheep ileal Peyer's patch, has a role in the generation of the primary B lymphocyte repertoire and the production of the systemic B lymphocyte pool. However, following ileal immunisation, a considerable F4-specific antibody response was induced (higher than following oral immunisation), and following intestinal immunisation (in jejunal, ileal and colonic lumen) a high number of F4-specific ASC was recovered from the ileal Peyer's patch. The latter seems to be in contrast to the less lymphocyte migration to the porcine ileal Peyer's patch observed in several studies (Binns and Licence, 1985; Rothkötter *et al.*, 1990, 1993). These findings seem not to fit with their role as primary

lymphoid organ. Indeed, differences were observed between the pig and the sheep ileal Peyer's pach. In the pig ileal Peyer's patch, the total number of follicles increased with age and their development was dependent on antigenic stimulation after birth (Rothkötter and Pabst, 1989), contrasting with the regression of the ileal Peyer's patch observed in lambs. Such increase in size is not characteristic for a primary lymphoid organ, though regression does eventually occur in older animals at about one year of age (Stokes *et al.*, 1994). Furthermore, in contrast to lambs, resection of the ileal Peyer's patch in newborn pigs did not result in a decrease of the B lymphocyte numbers in the blood (Pabst and Rothkötter, 1999). However, it is not known whether resection at a different time would result in a different outcome. Bianchi *et al.* (1992) even suggested that the ileal Peyer's patch is a secondary lymphoid tissue, with its development dependent on antigenic stimulation and with significantly higher number of T cells than in the sheep ileal Peyer's patch. Moreover, different studies demonstrate the uptake of particulates by the ileal Peyer's patch (Torché *et al.*, 2000; Gebert *et al.*, 1994). It further has to be stressed that the ileal patch also differs in subset composition, lymphocyte traffic and morphology; the last part before the ileocaecal junction resembles the jejunal patches (Binns and Pabst, 1988; Zuckermann and Gaskins, 1996). These findings together with our data, lead to the conclusion that the porcine ileal Peyer's patch surely not only functions as a primary lymphoid organ, but that besides its involvement in the generation of the primary B lymphocyte repertoire (Andersen *et al.*, 1999), it may be involved in the induction of mucosal immunity, albeit less efficiently compared to the jejunal Peyer's patches.

Following targeting of the F4 fimbriae to the lamina propria by local injection, an F4-specific intestinal immune response, although lower than following targeting to the jejunal Peyer's patches, was induced. This clearly demonstrates that although the lamina propria is the major mucosal effector site of the gut-associated immune system, immune responses may be induced at that site as discussed in *chapter 1* and *2*.

8.1.2. The involvement of enterocytes and M cells in the transepithelial transport of the F4 fimbriae and in the induction of the intestinal immune response in F4R$^{\pm}$ pigs

It is a remarkable finding that oral administration of F4 fimbriae induces an intestinal immune response, since soluble antigens mainly induce tolerance (McGhee *et al.*, 1992; Strobel and Mowat, 1998). However, in our laboratory it has been demonstrated that oral administration of F4 fimbriae to F4R^{-} pigs does not result in the induction of F4-specific ASC in the intestine, demonstrating the important role of the F4-specific receptors in the induction of the protective intestinal immune response in the F4R^{+} pigs (Van den Broeck *et al.*, 1999a). As discussed in *chapter 1*, the initial step for the induction of the intestinal immune response following oral administration is the ability of the antigen either to activate the epithelial cell or to penetrate and/or to cross the epithelial barrier after interaction with intestinal epithelium. Using intestinal loop systems, we demonstrated that in F4R^{+} pigs, the F4 fimbriae bind to villous enterocytes and to the cells of the follicle-associated epithelium (FAE), namely the M cells and the follicle-associated enterocytes of the jejunum and the ileum *(chapter 6)*. Furthermore, following F4R binding, the F4 fimbriae were endocytosed by the epithelial cells (villous enterocytes, M cells and follicle-associated enterocytes). F4 could further be identified in the intestinal LP or the Peyer's patch' dome region, demonstrating translocation of F4 across the epithelial layer. In contrast to the F4R^{+} pigs, the F4 fimbriae did not adhere to the intestinal epithelium of the F4R^{-} pig and endocytosis of the fimbriae could not be identified *(chapter 6)*. This demonstrates that binding of the F4 fimbriae to the F4-specific receptors is required for efficient uptake and subsequent translocation across the intestinal epithelial barrier. However, only one F4R^{-} animal was studied. Although, it is very likely that no *in vivo* binding will occur in all F4R^{-} pigs, more animals have to be studied to confirm this hypothesis. As described in *chapter 1*, binding to the apical membrane is an important determinant as to whether proteins are translocated across the epithelial cell, which is best obtained by receptor-mediated endocytosis. Receptor-mediated internalisation occurs within minutes and the rate of this uptake is many orders of magnitude higher than would be expected for bulk phase uptake or pinocytosis (Pryer *et al.*, 1992). Although in the

enterocytes, most of the endocytosed material is directed to lysomoses for degradation, a small part of the endocytosed compounds may be directed for transcytosis, depending on the targeting signals of the receptor, and may be shunted across the enterocyte away from lysosomal attack followed by release at the basolateral surface *(chapter 1)*. In *chapter 6*, we demonstrated that the F4 was in part transported across the epithelial layer. However, to what extent F4 is directed to and degraded in the lysosomes and whether the F4R itself mediates the endocytosis and transcytosis of F4 has to be elucidated further.

In contrast to the enterocytes, the M cells are specialised for transepithelial transport. Although their typical cell-surface characteristics, namely the spares microvilli, poorly developed glycocalix, the absence or reduction of overlying mucus and surface-associated secretory IgA, enhance the M cell interaction with luminal antigens, like for enterocytes, specific adhesion of the antigen with the apical membrane is necessary for efficient transport. This is reflected by the fact that bioadhesive particulate systems are taken up to a higher extent by M cells in comparison to uncoated particles, and explains the growing attention in these bioadhesive particulate systems for oral delivery of vaccines (Ponchel and Irache, 1998; Woodley, 2000; Clark *et al.*, 2000, 2002). In the $F4R^+$ pigs F4 did adhere to the apical membrane of the M cells of the jejunal and ileal Peyer's patches, after which it was endocytosed, whereas no endocytosis of the F4 fimbriae by the M cells was observed in the $F4R^-$ pig *(chapter 6)*. F4 binding to and endocytosis by M cells could be observed in the jejunal and ileal PP, indicating that the M cells of the ileal Peyer's patches are active in antigen uptake. However, although it is very likely that following endocytosis, the F4 fimbriae will be delivered in the intraepithelial pocket of the M cell, this could not be demonstrated. Further studies using transmission electron microscopy are necessary to confirm this. Using intestinal loops injected with yeast cells, Gebert *et al.* (1994) demonstrated that the number of yeast cells taken up in the jejunal PP far exceeded that in the ileal PP and suggested that this was attributed to the functional differences between these 2 types of PP. In *chapter 6*, no difference could be found in F4 binding to the FAE of the jejunal and ileal PP. However, difference in uptake of F4

between the FAE of these 2 types of PP can not be excluded since no quantification of F4 uptake was performed.

In *chapter 5*, a higher F4-specific antibody response in the intestine was induced following injection of the F4 fimbriae into the intestinal lumen as compared to injection in the LP, suggesting a role of the epithelium itself in the induced immune response. Although the porcine enterocytes do not express MHC class II molecules, making their role as antigen presenting cells very unlikely (Stokes *et al.*, 1996), their role in the induction of the protective immune response is probably not restricted to the transport of the antigen across the epithelial barrier. The enterocytes may augment the efficiency of subsequent antigen presentation by the professional antigen presenting cells present in the lamina propria (Vega-López *et al.*, 1993) by secretion of chemokines and cytokines (Kagnoff and Eckmann, 1997; Stadnyk, 2002) upon F4R binding. Furthermore, it is likely that by the F4R, the F4 is concentrated on the apical surface of the villous epithelium delaying the transit time of the F4 fimbriae down the small intestine. Consequently, it is possible that the F4 fimbriae are endocytosed and translocated gradually by the enterocyte, leading to release at the basolateral membrane for extended periods. This may mimic slow release or depot vaccine formulations leading to a better stimulation of the immune system (Gupta *et al.*, 1998b). This may also be true for the possible transport by the M cells. Indeed, using ligated loop systems, Pappo and Ermark (1989) demonstrated that the transcytosis of particles into the intraepithelial pocket by the M cells occurs in different rounds of endocytosis.

The fact that the jejunal Peyer's patches are the major inductive sites of the intestinal immune response *(chapter 5)* suggest that translocation of the F4 fimbriae across the FAE leads to better antigen presentation and activation of the immune system compared to uptake by villous enterocytes and release in the LP. As discussed in *chapter 1* and *2*, following uptake by the FAE, the antigen will be delivered in the dome region where APC, especially DCs are present in much larger numbers than in the LP. These DCs are capable of priming the naïve T cells present in huge numbers in the organized lymphoid compartment of the Peyer's patch.

In addition, in both F4R$^-$ as F4R$^+$ pigs, a comparable intestinal immune response was induced by targeting the F4 antigens to the lamina propria and the Peyer's patches via local injection *(chapter 5)*. This clearly demonstrates that the lack of F4-specific receptors and consequently the lack of efficient transepithelial transport are responsible for the absence of F4-specific ASC in F4R$^-$ pigs following oral administration of the F4 fimbriae.

8.1.3. A possible mechanism

Following oral or intestinal immunisation of F4R$^+$ pigs, the F4-specific receptors concentrate the F4 antigen on the epithelial surface, after which the F4 is gradually taken up and translocated. This results in release of the fimbriae at the basolateral membrane of the enterocytes or in the dome region of the PP for an extended period. Following release in the LP, the F4 antigen is internalised and processed by the many antigen-presenting cells present at that site (Vega-López *et al.*, 1993). These cells migrate to the mesenteric lymph nodes, where the intestinal immune response is induced and further developed, resulting in the recirculation of the primed lymphocytes (Bennell and Husband, 1981a, 1981b).

After endocytosis and translocation of the F4 by the FAE via enterocytes and possible M cells, the F4 antigen will directly be delivered in the dome region, where antigen-presenting cells and naïve T cells are present in huge numbers. Consequently, in contrast to delivery of the antigen into the LP, the mucosal immune system will be more efficiently induced, leading to higher amounts of F4-specific ASC in the Peyer's patches, mesenteric lymph nodes and lamina propria, the latter following recirculation of the primed lymphocytes.

In contrast to the F4R$^+$ pigs, the F4 fimbriae do not adhere to the intestinal epithelium and consequently not more than a relatively small amount of the F4 antigen will be captured in a non-specific way in the F4R$^-$ pigs, which is probably too low to induce F4-specific ASC in the intestine. On the other hand, it may be possible that the antigens that are endocytosed by non-specific mechanisms will be delivered into the lysosomes, causing loss of their immunogenicity when they ultimately are released at the basolateral plasma membrane, resulting in the absence of the F4-specific antibody

response following oral immunisation or in oral tolerance depending on the amount of ingested antigen. Furthermore, since data about the functional activities of the F4R are lacking, it is not known whether the F4 binding to the F4R itself induces immune system activating processes, such as the induction of proinfammatory cytokine and chemokine production by the epithelial cell. If this is the case, such immune stimulating processes will be absent following uptake by non-specific mechanisms in the $F4R^-$ pigs. Indeed, the enterocytes can produce and secrete a battery of cytokines of which some in response to certain luminal antigens. Since APCs are involved in the induction of either immunity or tolerance, the outcome of the antigen presentation is probably dependent on the appropriate secretion of cytokines or inflammatory signals. However, further research is needed to investigate the functional activities of the F4R.

8.2. THE PROTECTIVE CAPACITY OF THE INDUCED IMMUNE RESPONSE FOLLOWING IMMUNISATION OF SUCKLING PIGLETS WITH ENTERIC-COATED PELLETS OF F4 FIMRIAE

Since an active mucosal immunity is needed at the moment of weaning to prevent ETEC-induced postweaning diarrhoea, the protective capacity of the oral F4 immunisation of suckling piglets against an $F4^+$ETEC infection immediately postweaning was evaluated in *chapter 7*. In suckling piglets, the major portion of the F4 fimbriae will not be digested by gastric pepsin due to the higher gastric pH and faster gastric emptying compared to weaned pigs *(chapter 4)*. However, the F4 fimbriae may lose their antigenicity as a result of degradation by intestinal proteases present in the duodenum. Moreover the F4 fimbriae will be partially inactivated by milk-factors *(chapter 7)*. In order to overcome these obstacles, enteric-coated pellets containing the F4 fimbriae were developed. Hereto, the GI transit times of pellets *(chapter 3)* and pH *(chapter 4)* were studied. Furthermore, these enteric-coated pellets release the F4 fimbriae in the beginning of the jejunum and consequently bring the fimbriae closer to the major inductive sites, namely the jejunal PP *(chapter 5)*. Finally, such a vaccine formulation that can be mixed with creep feed will be more appropriate for regular administration to suckling animals,

compared to oral solution. An enteric coated formulation was evaluated for oral vaccination in *chapter 7*.

Oral vaccination with F4 fimbriae in PBS and in enteric-coated pellets were able to evoke an immune response in suckling piglets as significant F4-specific serum antibodies were developed. Compared to oral F4 administration in PBS, vaccination of the piglets with enteric-coated pellets of F4 resulted in a marginal but significant reduction in F4$^+$*E. coli* faecal excretion upon F4$^+$ETEC challenge. However, the colonisation of the small intestine with F4$^+$ETEC could not be prevented. This contrasts with oral F4 administration to pigs postweaning (Van den Broeck *et al.*, 1999a). This could be attributed to (i) the still young mucosal immune system of the suckling piglet (Roth, 1999; Pescovitz, 1998) causing a less efficient activation of the gut-associated immune system on immunisation (ii) higher susceptibility of the young 5-week-old newly weaned piglets to F4$^+$ETEC in comparison to the 11-week old pigs in the study of Van den Broeck *et al.* (1999a). Indeed, the first week postweaning piglets are extremely vulnerable to ETEC infections due to the predisposing factors involved in the postweaning diarrhea complex [physical, nutritional and social stress accompanying weaning, slower gastrointestinal transit *(chapter 3)*], which are absent in older pigs. Furthermore, in older pigs, higher resistance to F4$^+$ETEC infection may naturally develop due to the release of F4 receptors in the intestinal mucus layer with increasing age (Conway *et al.*, 1990).

8.3. MAIN CONCLUSION AND FUTURE PERSPECTIVES

In the present work the induction of the intestinal mucosal immune response following oral administration of F4 fimbriae was studied in more detail to allow efficient vaccine design against F4$^+$ETEC-induced postweaning diarrhoea. This work clearly demonstrated that the jejunal PP are the major inductive site of the intestinal immune system in young piglets, whereas the ileal PP are of lower importance. Moreover, there was abundant evidence that the F4 antigen is endocytosed by villous enterocytes and cells of the FAE, namely M cells and follicle-associated enterocytes, followed by its translocation across the intestinal epithelial barrier and further evidence is given that this transport following F4R binding is crucial in the induction of the F4-specific antibody

response in $F4R^+$ pigs. Data about GI transit time of pellets and GI pH were given allowing the development of solid vaccine formulations. An experiment in which newly-weaned piglets were challenged with $F4^+$ETEC after they were immunised during the suckling period with enteric-coated pellets of F4 that release the antigen in the beginning of the jejunum, showed that enteric-coating of the F4 fimbriae was beneficial but could not guarantee complete protection.

However, to develop a vaccine which can prevent postweaning diarrhoea, further research is needed to augment the activation of the still young immune system of the suckling piglets, thereby enhancing the protective capacity of the F4 vaccine. Different strategies can be used to accomplish this. (i) Since the jejunal PP are the major inductive sites of the intestinal F4-specific antibody response, selective targeting of the F4 fimbriae to these jejunal PP can enhance the intestinal immune response, leading to a higher efficacy of the vaccine. It has been shown that particles, nanoparticles to a greater extent over larger particles (Jenkins *et al.*, 1994; Desai *et al.*, 1996) are taken up by the M cells (Jepson *et al.*, 1996) and can be used for targeting antigens to the PP. However, to allow an efficient delivery of the antigen in the intraepithelial pocket by the M cells, specific adhesion to the apical membrane is necessary (O'Hagan, 1998; Brayden and Baird, 2001). Hereto, the particulates can be coated with bioadhesions (Ponchel and Irache, 1998). However, in contrast to other species, lectin binding glycolipids or -proteins specific for the porcine M cell apical surface have not been identified. Nevertheless, the expression of the IgA-specific receptor, mediating the transcytosis of sIgA from the intestinal lumen to the underlying gut-associated organised lymphoid tissue (Mantis *et al.*, 2002) may be conserved among species, including the pig and may be exploited for selective targeting to M cells. Zhou *et al.* (1995) and Velez *et al.* (1997) have already demonstrated that selective targeting to M cells can be achieved by coating carrier liposomes with IgA which further results in enhanced immune responses. Furthermore, the membrane protein β1-integrin, apically located on M cells, and exploited by pathogenic *Yersinia* via invasin binding in order to attach and invade the M cells (Clark *et al.*, 1998; Schulte *et al.*, 2000), may also be used for selective targeting to the M cells. However, it will have to be elucidated whether

selective targeting of the F4 fimbriae to the M cells by use of bioadhesive particulates will result in a higher uptake of the fimbriae compared to the F4R-dependent endocytosis and whether this will be able to enhance the immune response. (ii) The use of additionally adjuvants such as CT, LT, vitamin D3 and CpGs (Daynes *et al.*, 1996; Holmgren *et al.*, 2003; Van der Stede *et al.*, 2003) may lead to better activation of the gut-associated immune system. Moreover, these adjuvants may be incorporated together with the F4 fimbriae inside the enteric-coated pellets or microspheres. (iii) Modifications in the immunisation schedule with oral immunisation at different ages of the young piglet may lead to better immune induction. (iv) In addition to the oral immunisation, systemic immunisations (ID, SC, IM) with F4 or FaeG-coding DNA (Fischer *et al.*, 2003) and adjuvants that modulate the immune response towards the IgA type (vitamin D3 or CpGs) (Daynes *et al.*, 1996; Enioutina *et al.*, 2000; Van der Stede *et al.*, 2003) can be used to prime the intestinal mucosal immune system of suckling piglets more efficiently.

SUMMARY

Intestinal infections with enterotoxigenic *Escherichia coli* (ETEC) affect neonatal and recently weaned piglets. These infections cause diarrhoea and are responsible for severe economic loss due to growth retardation, elevated drug use and mortality. In general, most neonatal infections can be prevented by passive colostral and lactogenic immunity obtained by vaccination of the sow. However, this passive protection decreases with aging and disappears at weaning. As a consequence, the newly weaned piglet becomes highly susceptible to enteropathogens. In order to protect the newly weaned piglet, an active immunity is needed in the form of antigen-specific secretory IgA (sIgA) in the gut lumen. However, available parenteral vaccines stimulate the systemic (IgG antibodies) rather than the mucosal immune system. Alternatively, oral vaccines should be used to stimulate the intestinal mucosal immune system.

Competent oral veterinary vaccines for inducting mucosal protection are not yet available. Indeed, the oral route of delivery is the most challenging and difficult to exploit for proteins. The problems inherent with this route of delivery include: (i) low gastric pH and digestive enzymes causing degradation of the antigen; (ii) poor uptake of the antigen; and (iii) induction of oral tolerance instead of protective mucosal immunity by the gut-associated lymphoid tissue. Consequently, the antigen must not only survive the hostile gastric and intestinal intraluminal environments, it also has to interact with the intestinal epithelial cells and cross the epithelial barrier. However, interaction with the intestinal epithelial cells is hampered by the mucus gel layer, the cell surface glycocalyx and the closely packed microvilli, which not only act as a diffusion barrier, but also create a highly degradative microenvironment. Furthermore, to be effective as a vaccine, the antigen has to stimulate the intestinal immune system to produce a protective mucosal immunity. On the contrary, harmless antigens usually activate immunosuppressive mechanisms, resulting in oral tolerance.

Oral delivery systems can help to overcome these problems by reducing gastric and intestinal degradation of the antigen and by targeting the antigen to the specific

immunological induction site(s) of the gut-associated lymphoid tissue. By doing so, these delivery systems reduce the dose of antigen needed to induce a protective immune response.

F4 fimbriae bearing ETEC ($F4^+$ETEC) are one of the most prevalent isotypes causing postweaning diarrhoea. In our laboratory it has been demonstrated that the oral administration of purified F4 fimbriae to weaned piglets can induce protection against subsequent challenge with $F4^+$ETEC. However, to induce a protective intestinal immune response at weaning, the piglet has to be vaccinated during the suckling period. The use of oral delivery systems which protect the F4 fimbriae both against gastric degradation as well as against F4-neutralising milk factors and antibodies, and which subsequently deliver the F4 fimbriae at the immunologic induction site(s) in the gastrointestinal tract will allow the most efficient vaccination.

Chapter 1 reviews the present knowledge on the antigen transport across the intestinal epithelial barrier. After a short overview of the major cells of the villous epithelium, the morphological characteristics of the villous enterocytes were described in relation to their role in antigen uptake. The different mechanisms of transcellular and paracellular transport were given and the role of the enterocytes as antigen presenting cells was discussed. Furthermore, current knowledge on the role of dendritic cells in sampling of intestinal antigens was depicted. Moreover, the morphological features of the cells of the follicle-associated epithelium (FAE), namely the M cells and the follicle-associated enterocytes were described. The ontogeny of the FAE and M cells, and the M cell function with an emphasis on the direct transport of macromolecules to the immune cells of the Peyer's patches were discussed.

In *chapter 2* knowledge on the mucosal immune system of the gut with a major focus on the generation and function of secretory immunoglobulin A (sIgA) has been reviewed. A first part describes the organisation of the gut-associated lymphoid tissue into the inductive and effector sites. In the second part, the generation of sIgA, mechanisms of sIgA-mediated protection, and the distribution of the sIgA response were outlined. Furthermore, the role of B1 lymphocytes in the generation of sIgA was described. In the

final part of chapter 2, the importance of other antibodies, in addition to sIgA, in providing mucosal protection was discussed.

Chapter 3 to 7 presents the experimental work of the thesis. The objective was to examine the following questions:

- What are the gastrointestinal transit times of nondisintegrating pellets in suckling and recently weaned piglets?
- What is the gastrointestinal pH in suckling and recently weaned piglets? Are the F4 fimbriae degraded by the gastric pepsin or gastric and intestinal pH?
- Are the enterocytes or M cells responsible for the translocation of the F4 fimbriae across the intestinal epithelial barrier?
- At which sites in the gastrointestinal tract is the F4-specific immune response induced, and what is the major induction site: the lamina propria, the jejunal Peyer's patches or the ileal Peyer's patches?
- Can enteric coated pellets containing F4 fimbriae be used for oral vaccination against $F4^+$ETEC during the suckling period of the piglet as compared to F4 fimbriae in solution?

In *chapter 3*, the gastrointestinal (GI) transit time of nondisintegrating pellets in suckling and recently weaned piglets was determined, as transit times are essential criteria to develop oral vaccine delivery systems. Nondisintegrating radio-opaque pellets were used so that the transit times could be defined by radiography. The pellets were given orally and the radiographs were analysed with a software programme to calculate the number of pellets present in the different parts of the GI tract. In suckling piglets, the gastric emptying was faster (75% in 1.5 to 3.5 h) and the colonic accumulation (to maximal 73%) was greater than in weaned piglets (3 days, 2 and 3 weeks postweaning, 65% gastric emptying in 18 h, 75% in 17 h, and 75% in 7 h, respectively; maximal colonic accumulations of 48%). Immediately after weaning, the transit was markedly prolonged after which the transit times shortened with increased postweaning time (3 days, 2 and 3

weeks postweaning, 85% excretion in 175.5, 77, and 50.5 h, respectively). The long transit time immediately after weaning is attributed to the weaning process, which causes a lot of stress (social, nutritional, physiological and environment stress) that has an enormous influence on the GI motility. Indeed, three weeks postweaning, the transit was no longer affected by weaning and retention appeared to be restricted to the stomach and the colon, having retention times comparable to those of growing and adult pigs as reported in the literature. The variation in transit times at the different time points around weaning should be taken into account when designing an enteric coating for oral administration of vaccines to suckling and recently weaned piglets.

In *chapter 4*, the stability of the F4 fimbriae to different pH's as found in the gut of piglets and to gastric digestion was determined. Hereto, the gastrointestinal pH in suckling and recently weaned piglets had to be measured. In the stomach the lowest pH was found in the fundus gland region. Gastric pH values below 2.5 were not found in suckling piglets or at weaning, in contrast with 1 and 2 weeks postweaning. The pH along the first half of the small intestine and in the caecum was negatively correlated with age. Subsequently, the stability of F4 fimbriae to different pH's and to pepsin was assessed *in vitro*. The F4 fimbriae were stable to pH 1.5 and 2 for 2 hours, whereas longer incubation periods resulted in conversion of the multimeric forms into monomers. The F4 fimbriae were partially degraded by incubation for 15-30 min in simulated gastric fluid at pH 1.5 and 2, and completely digested from 3 hours onwards. At pH 3, the fimbriae maintained their antigenicity for at least 4 hours. The results demonstrate that gastric digestion will only have a limited impact on oral immunisation since liquid passes through the stomach relatively quickly (50 % within 2 hours). However, we previously demonstrated that the transit times are prolonged shortly after weaning. During the period shortly postweaning, it could be necessary to protect the F4 fimbriae against gastric digestion to obtain efficient oral immunisation of the piglets.

Chapter 5 demonstrated that the jejunal Peyer's patches are the major inductive sites of the F4-specific intestinal immune response, whereas the ileal Peyer's patches and the lamina propria are of less importance as inductive sites. Hereto, newly weaned pigs

were either immunised orally, in the lumen of the mid-jejunum, the ileum or the mid-colon with isolated F4 fimbriae after which the intestinal and serum antibody response was quantified by enumerating the F4-specific antibody secreting cells (ASC) in the small intestine and determining the F4-specific antibody titers in serum and in saponin extracts of the jejunal mucosa. Each immunisation increased similarly the F4-specific IgM and IgA titer in serum, except for the colonic immunisation which failed to evoke F4-specific serum antibodies. On the other hand, specific serum IgG titers were significantly higher following oral immunisation. Throughout the small intestine, the highest number of F4-specific ASC was found following jejunal immunisation, followed by ileal and oral immunisation. Colonic immunisation only induced F4-specific ASC in the ileum. Determination of the specific antibodies in saponin extracts of the jejunum demonstrated diffusion of serum antibodies into the intestinal LP. To elucidate the importance of the jejunal Peyer's patches (JPP), the jejunal lamina propria (LP) and the ileal Peyer's patches (IPP) in the induction of the immune response, the F4 fimbriae were selectively targeted to the JPP, jejunal LP or IPP by local injection in the intestinal wall. Similar but low specific serum antibodies were evoked following each immunisation. However, in the small intestine the highest number F4-specific ASC was found following immunisation in the JPP, whereas immunisation in the jejunal LP and IPP resulted in a similarly lower intestinal antibody response.

Chapter 6 demonstrated that the binding to the F4R mediates results in endocytosis and transcytosis of the F4 fimbriae across the intestinal epithelium. Indeed, immunohistochemical analysis of the sections of the different intestinal loops incubated with isolated F4 fimbriae, indicated that the F4 did not adhere to the intestinal epithelium in the $F4R^-$ pig. On the other hand, in the $F4R^+$ pigs, the F4 strongly adhered to the epithelium of the villi in the jejunal loops with and without Peyer's patches as well as in the ileum. Furthermore, the F4 strongly bound to the follicle-associated-enterocytes as well as to the M cells of the FAE of the jejunal and ileal Peyer's patches. Moreover, following F4R binding, endocytosis and transcytosis of the F4 occurred after which F4 was released in the small intestinal lamina propria and dome region of the jejunal and ileal

Peyer's patch, where it can activate the gut-associated immune system. Consequently, these results may explain the successful use of F4 as immunogen following oral administration to F4R$^+$ pigs.

Chapter 7 analysed whether oral immunisation of the suckling piglets with F4 fimbriae could protect the piglets shortly postweaning against oral challenge with F4$^+$ETEC and whether the use of enteric-coated pellets of the F4 fimbriae could improve this protection. Despite the induction of a serum antibody response upon oral administration of both F4 fimbriae in pellets as in solution, the colonisation of the small intestine by F4$^+$ETEC upon oral challenge could not be prevented in contrast to newly weaned piglets. This could be attributed to the still young mucosal immune system of the suckling piglet. However a significant reduction in faecal F4$^+$*E. coli* excretion was found in the piglets vaccinated with F4 fimbriae in pellets, indicating that the use of an enteric-coat which protects the F4 fimbriae against inactivation by milk factors and degradation by enzymes improves vaccination. Consequently, such enteric-coated pellets can be used as solid formulation, mixable with creep feed. However, further optimisations are required to develop a vaccine that can prevent postweaning diarrhoea.

The final chapter, *chapter 8*, presents the general discussion and conclusions with respect to the obtained results. It was clearly shown that the presence of the F4R on the intestinal epithelium plays a crucial role in the induction of the mucosal immunity. Binding to F4R, present on the enterocytes of the villous epithelium, on the follicle-associated enterocytes and M cells, results in endocytosis and transcytosis of the F4 fimbriae across the intestinal epithelium. Consequently, in the F4R$^+$ pigs following F4R binding, the F4 is delivered in the (i) dome region of the ileal and (ii) the jejunal Peyer's patches, and in (iii) the intestinal lamina propria after which the underlying gut-associated immune system can become activated. However, as the jejunal Peyer's patches are the major inductive sites of the F4-specific intestinal immune response, it is suggested that the induction occurs most efficiently following deliverance of the F4 in the dome region of the jejunal Peyer's patch. In the F4R$^-$ pigs, no endocytosis and translocation of F4 across the intestinal epithelium occurs, which may explain the absence of induction of

the mucosal immunity following oral F4 administration. Challenge experiments in which suckling animals orally vaccinated with F4 fimbriae or enteric-coated pellets of F4 fimbriae were infected with virulent F4$^+$ETEC showed that no complete protection could be obtained but that the faecal F4$^+$ *E. coli* excretion was reduced by the use of enteric coated pellets. In conclusion, enteric-coated pellets can be used as solid formulation, mixable with creep feed, for oral vaccination of piglets. However, further research is needed to augment the activation of the still young immune system of the suckling piglets so that complete protection against postweaning ETEC-induced diarrhoea can be guaranteed following vaccination. Different strategies may be used to augment the immune activation: (i) selective targeting of the F4 fimbriae to the jejunal Peyer's patches, the major inductive sites of the immune response, (ii) usage of additional adjuvants such as CT, LT, vitamin D3 and CpGs, (iii) modifications of the immunisation schedule or (iv) usage of combinations of oral immunisation and systemic immunisations, the latter together with adjuvants which can modulate the immune response towards IgA in order to obtain an effective priming of the intestinal mucosal immune system of the suckling piglet.

SAMENVATTING

Enterotoxigene *Escherichia coli* (ETEC) zijn een belangrijke oorzaak van intestinale infecties bij neonatale en pasgespeende biggen. Dergelijke infecties veroorzaken diarree en zijn verantwoordelijk voor zware economische verliezen ten gevolge van groeivertraging, verhoogd gebruik van geneesmiddelen en sterfte. Neonatale infecties met enteropathogenen kunnen voorkomen worden door een goede lactogene immuniteit, die o.a. verkregen kan worden door vaccinatie van de zeugen tijdens de dracht. Deze passieve maternale bescherming neemt af met de leeftijd en verdwijnt bij het spenen. Als gevolg hiervan worden de pasgespeende biggen opnieuw heel vatbaar voor infecties met enteropathogene bacteriën. Een actieve immuniteit is dan vereist om deze pasgespeende biggen te beschermen. Een protectieve immuniteit berust op de lokale productie van antigeenspecifieke antistoffen van het secretorisch IgA type (sIgA). Deze verhinderen, wanneer ze gesecreteerd worden in de darm, adhesie van de enteropathogene bacteriën aan het darmepitheel. Parenterale vaccins activeren in regel eerder het systemisch (IgG antilichamen) dan het mucosaal immuunsysteem, wat de effectiviteit van dergelijke vaccins voor de preventie van mucosale infecties verlaagt. Om het intestinaal mucosaal immuunsysteem te stimuleren, zouden orale vaccins eerder aangewezen zijn. Momenteel zijn er echter nog geen competente orale vaccins die een intestinale protectie induceren beschikbaar voor diergeneeskundig gebruik.

Van alle wegen om proteïnen toe te dienen is de orale weg de moeilijkste en vormt daarom een grote uitdaging. Volgende problemen zijn inherent aan deze toedieningsweg: (i) lage gastrische pH en verteringsenzymen in maag en darm die degradatie veroorzaken van het antigeen; (ii) zwakke opname van het antigeen; en (iii) inductie van orale tolerantie in plaats van een beschermende mucosale immuniteit door het darm-geassocieerd lymphoïd weefsel. Het antigeen moet bijgevolg niet alleen de sterk degraderende omgeving van de maag en de darm doorstaan, het moet ook in staat zijn de darmepitheelcellen te binden en de epitheelbarrière te doorkruisen. Interactie met de darmepitheelcellen wordt sterk bemoeilijkt door de aanwezigheid van de mucusgellaag,

de glycocalyx en de dicht op elkaar gepakte microvilli, die niet alleen als een diffussiebarrière fungeren, maar ook een heel degraderende micro-omgeving creëren. Om effectief te zijn als vaccin, moet het antigeen bovendien het intestinaal immuunsysteem stimuleren om een protectieve mucosale immuniteit te garanderen. Onschadelijke antigenen activeren immers in regel immunosuppressieve mechanismen die resulteren in orale tolerantie.

Orale toedieningssystemen kunnen helpen deze problemen te overwinnen door de degradatie van het antigeen in de maag en darm te voorkomen en door het antigeen te richten naar de specifieke immunologische inductieplaats(en) van het darm-geassocieerd lymfoïd weefsel.

ETEC stammen die F4 (K88) fimbriae bezitten, zijn één van de meest prevalente isotypes die speendiarree veroorzaken. Deze $F4^+$ETEC kunnen met behulp van de F4 fimbriae binden aan F4 receptoren (F4R) ter hoogte van de dunne darmepitheelcellen en vervolgens enterotoxines produceren met diarree als gevolg. De aan- of afwezigheid van de F4R is genetisch bepaald waarbij de F4R expressie het dominante kenmerk is. Enkel F4-receptor-positieve ($F4R^+$) dieren kunnen geïnfecteerd worden door $F4^+$ETEC. Onderzoek aan het Laboratorium voor Immunologie heeft aangetoond dat orale toediening van gezuiverde F4 fimbriae aan gespeende $F4R^+$ biggen volledige bescherming induceert tegen een $F4^+$ETEC infectie. Opdat de biggen een beschermende intestinale immuniteit zouden bezitten op het ogenblik dat ze gespeend worden, moeten de biggen gevaccineerd worden tijdens de zoogperiode. De meest efficiënte en effectieve vaccinatie zal verkregen worden wanneer orale toedieningsvormen gebruikt worden die (i) de F4 fimbriae beschermen tegen zowel degradatie in de maag als tegen F4-neutraliserende melkfactoren en antistoffen en die (ii) vervolgens de F4 fimbriae vrijstellen ter hoogte van de immunologische inductieplaats(en) in de darm.

Deel I van dit proefschrift *(hoofdstukken 1 en 2)* is een literatuurstudie die eerst antigeenopname doorheen de epitheelbarrière bespreekt *(hoofdstuk 1)*, en vervolgens het mucosaal immuunsysteem en IgA behandelt *(hoofdstuk 2)*. In een eerste deel van *hoofdstuk 1* worden, na een kort overzicht van de voornaamste celtypes van het

villusepitheel, de morfologische kenmerken van de villus enterocyten in relatie tot hun rol in antigeentransport beschreven. De verschillende mechanismen van transcellulair en paracellulair transport worden weergegeven en de rol van de enterocyten als antigeenpresenterende cellen wordt bediscussieerd. Verder wordt de huidige kennis over de rol van de dendrietcellen in de opname van intestinale antigenen vermeld. In een tweede deel worden de morfologische kenmerken van de cellen van het follikel-geassocieerd-epitheel (FAE), namelijk de M-cellen en de follikel-geassocieerde enterocyten weergegeven. De ontogenie van het FAE en de M-cellen, en de functie van de M-cel met een nadruk op het rechtstreekse transport van macromoleculen naar de immuuncellen van de Peyerse platen worden bediscussieerd.

In een eerste deel van *hoofdstuk 2* wordt de organisatie van het darm-geassocieerd lymphoïd weefsel in de inductie- en effectorplaatsen beschreven. In een tweede deel worden de generatie van sIgA, de mechanismen van de sIgA-gemedieerde bescherming, en de distributie van de sIgA respons weergegeven. Verder wordt de rol van de B1 lymfocyten in de generatie van sIgA toegelicht. In een laatste deel van dit hoofdstuk, wordt het belang van andere antistoffen dan sIgA, in de mucosale bescherming bediscussieerd.

Deel III (hoofdstukken 3 tot 7) bevat het eigen onderzoek. Hierbij werden de volgende vraagstellingen onderzocht (Deel II doelstellingen):

- Wat zijn de gastrointestinale transittijden van niet-desintegrerende pellets in zuigende en pasgespeende biggen?
- Wat is de gastrointestinale pH in zuigende en pasgespeende biggen? Worden de F4 fimbriae afgebroken door gastrisch pepsine en/of door de pH van de maag en darm?
- Staan de enterocyten of de M-cellen in voor de translocatie van de F4 fimbriae doorheen de intestinale epitheelbarrière?
- Op welke plaatsen in de gastrointestinale tractus wordt de F4-specifieke immuunrespons geïnduceerd, en wat is de belangrijkste inductieplaats: de lamina propria, de jejunale Peyerse platen of de ileale Peyerse platen?

- Kunnen enterisch omhulde pellets van F4 fimbriae gebruikt worden om zuigende biggen oraal te vaccineren tijdens de zoogperiode tegen F4$^+$ETEC en geven dergelijke pellets een betere bescherming in vergelijking met F4 fimbriae in oplossing?

In *hoofdstuk 3* werden de gastrointestinale transittijden van niet-desintegrerende pellets bij zuigende en pasgespeende biggen bepaald. Transittijden zijn immers essentiële criteria voor de ontwikkeling van toedieningsvormen voor orale vaccins. Niet-desintegrerende radio-opake pellets werden gebruikt zodat de transittijden radiografisch konden worden bepaald. De pellets werden oraal toegediend en de radiografieën werden geanalyseerd met een computerprogramma om het aantal pellets, aanwezig in de verschillende delen van de GI tractus te berekenen. Bij zuigende biggen was de maaglediging sneller (een maaglediging van 75% in 1.5 tot 3.5 uur) en de accumulatie in het colon (tot 73%) groter dan bij de gespeende biggen (3 dagen, 2 en 3 weken na het spenen was respectievelijk 65% van de maag geledigd na 18 uur, 75% na 17 uur, en 75% na 7 uur; de accumulatie in het colon was maximaal 48%). Onmiddellijk na het spenen was de transit opmerkelijk verlengd waarna de transittijden opnieuw verkortten (3 dagen, 2 en 3 weken na het spenen was respectievelijk 85% van de pellets uitgescheiden in 175.5, 77, en 50.5 uur). De verlengde transit kan toegeschreven worden aan het spenen dat veel stress veroorzaakt (sociale, nutritionele, fysiologische, psychologische stress en omgevingsstress) wat een enorme invloed heeft op de GI motiliteit. Drie weken na het spenen werd de transit niet langer beïnvloed door het spenen en bleef de retentie van de pellets beperkt tot de maag en het colon. De retentietijden bij de 3 weken gespeende biggen waren vergelijkbaar met deze van groeiende en volwassen varkens zoals beschreven in de literatuur. De variatie in transittijden op de verschillende ogenblikken rond het spenen moet in acht genomen worden bij de ontwikkeling van een enterische omhulling voor orale toediening van vaccins aan zuigende en pasgespeende biggen.

In *hoofdstuk 4* werd de stabiliteit van de F4 fimbriae bepaald tegenover verschillende zuurtegraden, zoals deze aangetroffen worden in de darm van biggen, en

tegenover vertering door gesimuleerd maagsap. Eerst werd de gastrointestinale pH in zuigende en pasgespeende biggen gemeten. De laagste pH-waarden in de maag werden teruggevonden in de fundus klierregio. In de maag van zuigende biggen en biggen op de dag van het spenen werden geen pH-waarden lager dan 2.5 teruggevonden, in tegenstelling tot waarden bij biggen die 1 en 2 weken gespeend waren. In de eerste helft van de dunne darm en in het caecum was de pH negatief gecorreleerd met de leeftijd. Vervolgens werd de stabiliteit van de F4 fimbriae tegenover verschillende pH's en pepsine bepaald *in vitro*. De F4 fimbriae waren bij een pH van 1.5 en 2 stabiel gedurende 2 uur, terwijl langere incubatieperioden resulteerden in een conversie van de multimere vormen in monomeren. De F4 fimbriae waren gedeeltelijk afgebroken na incubatie gedurende 15 tot 30 minuten in gesimuleerd maagsap bij een pH van 1.5 en 2, en waren volledig verteerd vanaf een incubatieperiode van 3 uur. Bij een pH van 3 behielden de fimbriae hun antigeniciteit voor ten minste 4 uur. De resultaten tonen aan dat vertering in de maag slechts een gelimiteerde impact zal hebben op orale immunisatie aangezien vloeistoffen vrij vlug uit de maag worden geledigd (50% in 2 uur). Kort na het spenen zijn de transittijden echter aanzienlijk verlengd. Tijdens de periode kort na het spenen kan het bijgevolg nodig zijn de F4 fimbriae te beschermen tegen vertering in de maag om de biggen efficiënt te vaccineren.

Hoofdstuk 5 toonde aan dat de jejunale Peyerse platen de belangrijkste inductieplaatsen zijn van de F4-specifieke intestinale immuunrespons, terwijl de ileale Peyerse platen en de lamina propria van minder belang zijn als inductieplaats. Pasgespeende biggen werden oraal geïmmuniseerd of geïmmuniseerd in het lumen ter hoogte van het midden van het jejunum, het ileum of het midden van het colon. Vervolgens werd bepaald welke van deze immunisaties de meeste antistoffen genereerde in de dunne darm. Hiertoe werden de F4-specifieke antistoffensecreterende cellen (ASC) in de dunne darmwand geteld met behulp van ELIspot-testen, en werd de F4-specifieke antistoffentiter in serum en saponine extracten van het jejunum bepaald met behulp van ELISA. Elke immunisatie verhoogde in gelijke mate de F4-specifieke IgM en IgA titer in het serum, met uitzondering van de immunisatie in het colon die niet resulteerde in de

inductie van F4-specifieke serumantistoffen. Daarentegen waren de F4-specifieke serum IgG titers beduidend hoger na orale immunisatie. Over de volledige lengte van de dunne darm werd het hoogste aantal F4-specifieke ASC teruggevonden na immunisatie in het jejunum, gevolgd door ileale en orale immunisatie. Na immunisatie in het colon bleven de F4-specifieke ASC beperkt tot het ileum. Bepaling van de specifieke antistoffen in saponine extracten van het jejunum toonde diffusie aan van serumantistoffen in de lamina propria van de darm. Om het belang van de jejunale Peyerse platen (JPP), de jejunale lamina propria (LP) en de ileale Peyerse platen (IPP) in de inductie van de immuunrespons op te helderen, werden de F4 fimbriae selectief gericht naar de JPP, de jejunale LP, of de IPP door de F4 fimbriae lokaal in de darmwand te injecteren. Specifieke serumantistoffen werden in lage hoeveelheden en in gelijke mate geïnduceerd na iedere immunisatie. Het hoogste aantal F4-specifieke ASC in de dunne darm werd daarentegen teruggevonden na immunisatie in de JPP, terwijl immunisaties in de jejunale LP en IPP resulteerden in een lagere intestinale antistoffen respons.

Hoofdstuk 6 toonde aan dat de binding aan de F4-receptor (F4R) resulteert in endocytose en transcytose van de F4 fimbriae doorheen het darmepitheel. Immunohistochemische analyse van secties van verschillende darmlussen die waren geïncubeerd met geïsoleerde F4 fimbriae, toonde aan dat de F4 fimbriae niet binden aan het darmepitheel een F4R-negatieve ($F4R^-$) big. In F4R-positieve ($F4R^+$) biggen binden de F4 fimbriae in even sterke en gelijke mate aan het villusepitheel in jejunale darmlussen dan aan de villi van de mesenteriale en anti-mesenteriale zijde in darmlussen ter hoogte van de jejunale en ileale Peyerse platen. Verder binden de F4 fimbriae sterk aan de follikel-geassocieerde enterocyten en M-cellen van het FAE van de jejunale en ileale Peyerse platen. Bovendien vond na de binding aan de F4R endocytose en transcytose van de F4 fimbriae plaats, waarna de fimbriae werden vrijgesteld in de lamina propria van de dunne darm en de dome regio van de jejunale en ileale Peyerse platen. Daar kunnen ze het darm-geassocieerd immuunsysteem activeren. Dit kan bijgevolg het succes van F4 als immunogeen na orale toediening aan $F4R^+$ biggen verklaren.

In *hoofdstuk 7* werd nagegaan of orale immunisatie van zuigende biggen met F4 fimbriae bescherming kon bieden tegen orale infectie met F4$^+$ETEC kort na het spenen en of het gebruik van enterisch omhulde pellets die de F4 fimbriae bevatten de bescherming kon verbeteren. Ondanks de inductie van een serumantistoffenrespons na orale toediening van zowel de F4 fimbriae in oplossing als de enterisch omhulde pellets, kon de kolonisatie van de dunne darm door F4$^+$ETEC niet voorkomen worden. Dit in tegenstelling tot 11-weken oude biggen die oraal werden gevaccineerd na het spenen. Dit kan toegeschreven worden aan het jong mucosaal immuunsysteem van de zuigende big. Nochtans werd een significante reductie in fecale excretie van F4$^+$*E. coli* teruggevonden in de biggen die werden gevaccineerd met F4 fimbriae in pellets. Dit toont aan dat het gebruik van een enterische omhulling die de F4 fimbriae beschermt tegen inactivering door melkfactoren en tegen degradatie door enzymen de vaccinatie verbetert. Dergelijke enterisch omhulde pellets kunnen bijgevolg gebruikt worden als een vaste formulatie die mengbaar is met het voeder. Verdere optimalisaties zijn echter nodig om een vaccin te ontwikkelen dat volledige bescherming biedt tegen speendiarree.

Deel IV *(hoofdstuk 8)* bevat de algemene discussie en de belangrijkste conclusies. Dit werk heeft duidelijk aangetoond dat de aanwezigheid van F4R op het apicaal membraan van de darmepitheelcellen een cruciale rol speelt in de inductie van de F4-specifieke mucosale immuniteit. Deze F4R worden tot expressie gebracht door zowel de enterocyten van het villusepitheel als door de follikel-geassocieerde enterocyten en M-cellen van het FAE. Na binding van de F4-fimbriae aan deze F4R, treedt endocytose en transcytose van de F4 fimbriae op doorheen het darmepitheel. Vervolgens wordt het F4 vrijgesteld in (i) de dome regio van de ileale en (ii) de jejunale Peyerse platen, en (iii) in de intestinale lamina propria waar F4 het darm-geassocieerd immuunsysteem activeert. Het feit dat de jejunale Peyerse platen de belangrijkste inductieplaatsen zijn van de F4-specifieke intestinale immuunrespons, suggereert dat de inductie het meest efficiënt gebeurt na vrijstelling van het F4 in de dome regio's van de jejunale Peyerse platen. In de F4R$^-$ big kon geen endocytose en translocatie van F4 doorheen het darmepitheel aangetoond worden. Dit kan de afwezigheid van de mucosale immuniteit in F4R$^-$ biggen

verklaren na orale toediening van F4. Challenge-experimenten waarbij biggen geïnfecteerd worden met de virulente F4$^+$ETEC, nadat ze vooraf tijdens de zoogperiode oraal gevaccineerd werden met F4 fimbriae of met enterisch omhulde pellets van deze F4 fimbriae, toonde aan dat geen volledige bescherming, maar toch een verminderde kolonisatie kon bekomen worden door gebruik te maken van enterisch omhulde pellets. Enterisch omhulde pellets kunnen bijgevolg gebruikt worden als een vaste formulatie, die mengbaar is met het voeder, om biggen oraal te vaccineren. Verder onderzoek is echter nodig om het jonge immuunsysteem van de zuigende big beter te activeren zodat een volledige bescherming tegen ETEC-geïnduceerde speendiarree kan gegarandeerd worden na vaccinatie. Verschillende strategieën kunnen gevolgd worden om tot een betere activering te komen: (i) selectief richten van de F4 fimbriae naar de jejunale Peyerse platen, de belangrijkste inductieplaatsen van de immuunrespons, (ii) gebruik van bijkomende adjuvantia zoals CT, LT, vitamine D3 en CpGs, (iii) wijzigingen in het immunisatieschema of (iv) gebruik van combinaties van orale en systemische immunisaties met adjuvantia die de immuunrespons moduleren naar een IgA type respons, om het intestinaal mucosaal immuunsysteem van de zuigende big optimaal te primen.

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CURRICULUM VITAE

Veerle Snoeck werd geboren op 30 april 1977 te Gent. In 1995 beëindigde zij haar secondaire opleiding, richting Latijn-Wiskunde, aan het Instituut Zusters Maricolen te Deinze, In datzelfde jaar begon zij de studies Farmaceutische Wetenschappen aan de Universiteit van Gent, waar zij in 1997 het diploma van Kandidaat Apotheker behaalde. Vervolgens zette zij haar studies verder aan de Faculteit Wetenschappen, richting Biotechnologie, waar ze afstudeerde als Licentiaat in de Biotechnologie met grootste onderscheiding in 1999. In 2000 ontving zij een doctoraatsbeurs van het Bijzonder Onderzoeksfonds van de Universiteit Gent in the kader van een Geconcentreerde Onderzoeksactie (GOA) voor onderzoek naar vaccinontwikkeling tegen $F4^+$ enterotoxigene *Escherichia coli* infecties bij biggen. Dit onderzoek werd uitgevoerd aan het laboratorium voor Immunologie van de Huisdieren onder leiding van Prof. Dr. E. Cox en Prof. Dr. B.M. Goddeeris en leidde tot dit proefschrift. Tevens behaalde zij in 2003 het getuigschrift voor Doctoraatsopleiding in de Diergeneeskundige Wetenschappen. Veerle is auteur of mede-auteur van meerdere wetenschappelijke publicaties.

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