



Published in final edited form as:

*Immunol Invest.* 2010 ; 39(0): 383–406. doi:10.3109/08820131003622635.

## Secretory IgA: Arresting Microbial Pathogens at Epithelial Borders

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### Abstract

Secretory IgA (SIgA), the predominant class of antibody found in intestinal secretions. While SIgA's role in protecting the intestinal epithelium from the enteric pathogen and toxins has long been recognized, surprisingly little is known about the molecular mechanisms by which this is achieved. The present review summarizes the current understanding of how SIgA functions to prevent microbial pathogens and toxins from gaining access to the intestinal epithelium. We also discuss recent work from our laboratory examining the interaction of a particular protective monoclonal IgA with *Salmonella* and propose, based on this work, that SIgA has a previously unrecognized capacity to directly interfere with microbial virulence at mucosal surfaces.

### 1. Vulnerability of the intestinal epithelium to microbial infections

As the largest mucosal surface in the human body, the intestinal epithelium is highly vulnerable to microbial attack. Indeed, infections acquired through the consumption of contaminated food and water are a leading cause of morbidity and mortality worldwide. Children under the age of 5, in developing countries, are especially susceptible to such infections caused by rotavirus (Franco et al., 2006), *Shigella* species (Levine et al., 2007), and *Salmonella enterica* (Petri et al., 2008). Infections caused by *S. enterica* serovar Typhi (*S. Typhi*) alone are estimated to cause more than 16 million cases of typhoid fever, and more than half a million deaths, each year (Levine, 2006). In developed countries such as the United States, *Escherichia coli* O157:H7 and *S. enterica* serovar Typhimurium (*S. Typhimurium*) infections are increasingly associated with food processing and handling, and they therefore represent an emerging public health threat (Maki, 2009). In 2009, for example, an outbreak of *S. Typhimurium* associated with contaminated peanut butter affected hundreds of persons and resulted in at least 8 deaths. Additionally, many enteric pathogens including *S. Typhimurium*, as well as bacterium- and plant-derived toxins, are considered by the Centers for Disease Control and Prevention (CDC) as bioterrorism agents that could be (or have already been) deliberately used to contaminate food and water supplies with the intent of inducing illness at a local, regional or national level (Mantis et al., in press).

The intestinal epithelium is equipped with a number of innate defense mechanisms, to deter microbial pathogens and toxins from gaining access to epithelial cells receptors (Mantis and Bry, 2008; Turner, 2009). First, the epithelium itself is a barrier, consisting of a single layer of columnar epithelial cells arranged side-by-side and joined at their apical aspects by tight junctions. In the small intestine, absorptive enterocytes are the predominant epithelial cell

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type, and are responsible for the bulk of nutrient absorption from the gut lumen. The apical surfaces of these cells consist of rigid, closely packed microvilli rich in membrane-bound glycolipids and stalked glycoproteins, some of which are known receptors for pathogens (e.g., rotavirus) and toxins (e.g., cholera toxin, or CT). The tips of these microvilli are coated with a 400–500 nm thick glycoprotein meshwork known as the filamentous brush border glycocalyx (FBBG), which is proposed to function in epithelial defense by limiting the access of virus- and bacterium-sized particles to membrane-bound receptors (Frey et al., 1996; Mantis et al., 2000). Second, interspersed with the adsorptive enterocytes are a number specialized epithelial cell types that assist in innate immunity, including mucin-producing goblet cells and crypt-situated Paneth cells, which produce antimicrobial compounds including lysozyme and alpha-defensins (Deplancke and Gaskins, 2001; Lievin-Le Moal and Servin, 2006; Ouellette, 2006). Finally, epithelial cells, upon infection or toxin exposure, “call for help” by secreting pro-inflammatory chemokines and lipid mediators that trigger the recruitment and activation of leukocytes (e.g., polymorphonuclear cells) that aid in the phagocytosis of extracellular pathogens (Tam et al., 2008).

However, the innate immune system is of only limited efficacy in deterring the most virulent pathogens and toxins. *S. Typhimurium*, for example, defends itself against antimicrobial compounds in the intestinal lumen by altering its own gene expression, reducing permeability of its outer membrane, and activating extracellular scavenging pathways (Bader et al., 2003; Haraga et al., 2008; Raffatellu et al., 2009). Certain enterotoxins, of both plant and microbial origins, are so potent that even a small number of molecules are sufficient to induce epithelial dysfunction and/or host cell death (Mantis, 2005; Matsumura et al., 2007). Immunity against these agents necessitates the involvement of the body’s adaptive immune system, specifically the gut-associated lymphoid tissues (GALT). In the small intestine, the GALT consists of aggregates of lymphoid follicles known as Peyer’s patches (Fig. 1). These organized lymphoid follicles contain germinal centers enriched in B cells whose differentiation and somatic cell hypermutation are driven in response to antigens present in the intestinal lumen (Brandtzaeg and Johansen, 2005; Cerutti and Rescigno, 2008; Suzuki and Fagarasan, 2009). By virtue of the local cytokine environment, B cells within the Peyer’s patches not only undergo class switch recombination preferentially to IgA, but they also express J chain, the 15 kDa protein that serves to promote immunoglobulin dimerization (Brandtzaeg, 1974; Brandtzaeg and Johansen, 2005). As a result, mucosa-derived plasma cells secrete IgA in a dimeric or polymeric form.

The epithelium overlying organized lymphoid follicles, the so-called follicle-associated epithelium (FAE), is an integral component of the GALT (Fig. 1). Probably the most distinctive feature of the FAE is the presence of M cells, a unique epithelial cell type that is specialized for the uptake and transepithelial transport of particulate antigens, including macromolecules (Neutra et al., 1987; Pappo and Ermak, 1989), viruses (Amerongen et al., 1991; Sicinski et al., 1990; Wolf et al., 1981), bacteria (Jones et al., 1994; Owen et al., 1986; Sansonetti et al., 1996), and parasites (Marcial and Madara, 1986). Indeed, M cells have been considered the ‘gateway’ to the GALT (Neutra et al., 2001). The apical and basolateral surfaces of M cells have distinctive features that enable these cells to rapidly and efficiently deliver mucosal antigens from the lumen to underlying lymphoid follicles. For example, M cells lack the well-developed brush border and FBBG that is present on enterocytes. Consequently, M cell apical membranes are more accessible to particles, viruses, and bacteria than are the apical surface of adjacent enterocytes (Frey et al., 1996; Mantis et al., 2000). In mice and humans, the apical surfaces of M cells have a pattern of glycosylation that is distinct from that seen on the FAE enterocytes and villus enterocytes (Clark et al., 1993; Giannasca et al., 1994; Giannasca et al., 1999). M cells also selectively express an IgA receptor, as well as Toll-like receptors and other pattern recognition receptors on their apical membranes: such receptors probably facilitate antigen recognition and contribute to

signaling in the local environment (Chabot et al., 2006; Lo et al., 2003; Mantis et al., 2002; Tohno et al., 2005; Weltzin et al., 1989).

## 2. SIgA: Structure, production and transepithelial transport

Dimeric IgA secreted by plasma cells in the lamina propria is transported across the epithelium into intestinal secretions by the polymeric immunoglobulin receptor (pIgR). The pIgR is a transmembrane glycoprotein expressed exclusively on the basolateral surfaces of columnar epithelial cells, especially those in the crypts (Kaetzel, 2005; Mostov, 1994). The extracellular region of pIgR consists of five immunoglobulin-like domains that recognize dimeric IgA (and IgM). The receptor, in complex with dimeric IgA, is internalized by endocytosis at the cell's basolateral surface, and is then transported vectorially to the apical membrane. Once facing the intestinal lumen, the receptor is proteolytically cleaved, such that the 70 kDa extracellular domain of pIgR, known as secretory component (SC), is released in covalent association with IgA, giving rise to SIgA. Thus, SIgA is effectively a polypeptide complex that consists of two (or more) IgA monomers joined at their C-termini by J chain in association with SC. SC has a number of proposed functions besides assisting with the transport and release of IgA into the intestinal lumen; these functions include protecting IgA from proteolytic degradation and anchoring IgA in the mucus layer overlying the mucosal surfaces (see below) (Crottet and Corthesy, 1998; Ma et al., 1998; Phalipon et al., 2002; Phalipon and Corthesy, 2003).

Whereas mice have a single type of IgA, humans have two distinct IgA subclasses, IgA1 and IgA2. The primary difference between the two subclasses occurs in the hinge region between domains C<sub>1</sub> and C<sub>2</sub>: IgA1 has a 20-amino acid hinge whereas IgA2 has a 7-amino acid hinge (Mestecky et al., 2005). Mouse IgA has a short hinge region and most closely resembles human IgA2. To date, no differences in specific effector functions have been ascribed to the two human IgA subclasses. IgA1 and IgA2 are found in roughly equal proportions in secretions, except in the large intestine where IgA2 is more prevalent (Crago et al., 1984; Mestecky and Russell, 2009).

Human IgA1, IgA2, and SC are heavily glycosylated, a characteristic which is integral to their capacity to function in intestinal secretions. Human IgA1 has two *N*-glycosylation sites, and nine potential O-glycosylation sites within the serine- and threonine-rich hinge between C<sub>1</sub> and C<sub>2</sub> (Royle et al., 2003; Tomana et al., 1976). Human IgA2 has three (and in some isoforms, four) N-linked side chains, but no O-linked side chains. On average, oligosaccharide side chains account for ~10% of the molecular mass of the IgA. In contrast, glycans comprise only ~3% of the mass of human IgG. SC has seven *N*-glycosylation sites and approximately 15% of the molecular mass of the molecule is attributable to carbohydrates side chains, containing bi- and tri-antennary and Lewis-type structures (Hughes et al., 1999; Perrier et al., 2006). Recent molecular modeling of SIgA vividly demonstrates the spatial extent to which N- and O-linked glycan side chains project from Fc and SC, rendering them highly accessible to lectins and viral or bacterial adhesins (Royle et al., 2003). In marked contrast, the glycans on IgG are proposed to face inward and to be sequestered between the H chains (Mattu et al., 1998).

## 3. SIgA's mechanisms for protecting the epithelium

The present review will summarize the current understanding of how SIgA functions to prevent microbial pathogens and toxins from gaining access to the intestinal epithelium. Our focus will be limited to SIgA's activities within the intestinal lumen; we will not discuss the capacity of IgA to neutralize pathogens intracellularly during transepithelial transport (Burns et al., 1996; Corthesy et al., 2006; Fernandez et al., 2003; Mazanec et al., 1995; Mazanec et al., 1992), or to promote excretion of antigens present in the lamina propria (Kaetzel et al.,

1991; Kaetzel et al., 1994). Specific examples of two well-established mechanisms by which SIgA inhibits toxin, virus and bacterial contact with epithelial cells will be given. Last, we will discuss recent work from our laboratory in which we have demonstrated that a protective monoclonal IgA, known as Sal4, has the capacity to directly interfere with *S. Typhimurium* virulence factors, and effectively “arrest” the bacterium at the epithelial border.

### Blocking attachment to epithelial cell receptors

SIgA is capable of interfering with the earliest steps in the infection process by binding to epitopes on the surfaces of pathogens and toxins involved epithelial receptor recognition (Apter et al., 1993a; Mantis et al., 2006; Stubbe et al., 2000) (Fig. 1). SIgA’s ability to block epithelial receptor interactions can occur through antibody variable region (Fv)-dependent as well as Fv-independent mechanisms, as will be discussed below.

Probably the best example of Fv-dependent interference involves the interaction of SIgA with cholera toxin (CT). CT is a member of the A–B family of enterotoxins, which includes botulinum neurotoxins (BoNT), shiga toxins (Stx), and the plant-derived toxin, ricin. CT is the toxin primarily responsible for the secretory diarrhea associated with *Vibrio cholerae* infection (Butler and Camilli, 2005). The toxin’s A subunit (CTA) catalyzes the NAD-ribosylation of the regulatory GTPase,  $G_{s}$ , which in turn activates adenylate cyclase and cyclic AMP-dependent chloride secretion in crypt epithelial cells (Lencer and Tsai, 2003). The B subunit (CTB) oligomerizes to form a pentamer that binds specifically to the ganglioside GM<sub>1</sub>, and promotes toxin internalization. The toxin then traffics in a retrograde manner from the plasma membrane to the endoplasmic reticulum (ER), after which CTA is retrotranslocated into the cytoplasm (Lencer and Tsai, 2003). The effects of CT on intestinal epithelial cells can be studied in vitro using well-differentiated human intestinal cell lines such as T84 (Lencer et al., 1992).

It is now well established that SIgA is required for immunity to CT, and that protection is mediated primarily by antibodies that block toxin attachment to the epithelial cell receptor GM<sub>1</sub>. The requirement for SIgA in conferring immunity to CT was first demonstrated experimentally in a vaccine setting by Lycke and colleagues, who reported that J-chain knockout mice, following vaccination with CT, remained vulnerable to the effects of the toxin, whereas wild type control animals were immune (Lycke et al., 1999). Because J chain knockout mice had wild-type levels of anti-toxin IgA-producing B cells in the lamina propria, but severely reduced levels of SIgA levels in the intestinal lumen, it was concluded that antibodies in secretions were essential for full protection against the effects of CT, at least in the mouse model employed in this study. This conclusion was further supported by Uren and colleagues who reported a number of years later that CT-vaccinated pIgR knockout mice, which are effectively devoid of SIgA but have normal to elevated levels of IgA in serum, were susceptible to cholera toxin challenge (Uren et al., 2005).

To investigate the mechanism by which the SIgA protects the epithelium from CT, Apter and colleagues produced a collection of anti-toxin monoclonal IgA antibodies from the Peyer’s patches of CT-immunized mice (Apter et al., 1993a). Three anti-CTB dimeric IgA MABs were characterized in detail, and each was shown to block CT attachment to the apical surfaces of T84 cell monolayers in vitro. The three MABs were also capable of functioning in vivo, as evidenced by the fact that neonatal mice treated passively with the MABs were immune to CT-induced secretory diarrhea, weight loss and death (Apter et al., 1993b). It was proposed that the MABs did not directly interact with the GM<sub>1</sub> binding site on CTB, but, rather, functioned by steric hindrance. This conclusion was based on the observation that purified GM<sub>1</sub>, when added exogenously in an ELISA, did not competitively inhibit the antibodies from recognizing CTB.

SIgA has also been shown to prevent viral infections by blocking virus adhesion to epithelial cells. One notable example involves reovirus type 1 Lang (T1L), a murine enterovirus that initially infects the intestinal mucosa via attachment to Peyer's patch M cells (Wolf et al., 1981). Silvey and colleagues demonstrated that SIgA is required for full protection against reovirus, a conclusion based on the observation that IgA knockout mice are susceptible to secondary intestinal infections with reovirus, whereas wild type animals are immune (Silvey et al., 2001). To investigate the molecular mechanism underlying SIgA-mediated immunity to reovirus, Hutchings and colleagues examined the capacity of monoclonal IgA antibodies directed against viral surface antigens, including an adhesin and the capsid, to protect mice against oral T1L challenge (Hutchings et al., 2004). It was determined that protection was conferred by only one of the monoclonal antibodies tested, known as 1E1. 1E1 was determined to bind to the  $\alpha 1$  protein, an adhesin fiber known to promote viral attachment to a number of epithelial cell types, including M cells (Helander et al., 2003). The epitope recognized by 1E1 was localized to the receptor-binding head domain of  $\alpha 1$  (Helander et al., 2004). Monoclonal IgA antibodies against the other viral surface antigens, including the capsid protein, were not protective in the T1L peroral challenge model (Hutchings et al., 2004). The above studies demonstrate that protection against enteropathogenic viruses such as reovirus is conferred most effectively by SIgA antibodies directed against epitopes on the viral surfaces involved in attachment to epithelial cell receptors.

### Fv-independent blocking of pathogen attachment to the epithelium

SIgA is also capable of preventing pathogen and toxin attachment to epithelial surfaces, independent of the antibody variable region. In this respect, SIgA can be considered a component of the innate immune system. As noted above, both the IgA H chain (Fc) and SC are heavily glycosylated. Because the oligosaccharide side chains on SIgA share a high degree of similarity with those on the luminal face of the intestinal epithelium, it has been proposed that both IgA and SC can effectively serve as competitive inhibitors ("decoys") of pathogen binding to host cells (Dallas and Rolfe, 1998; Mantis et al., 2004; Mestecky and Russell, 2009; Perrier et al., 2006; Royle et al., 2003; Schroten et al., 1998; Wold et al., 1990).

This role for SIgA was recently demonstrated in the case of ricin toxin (Mantis et al., 2004). The B subunit (RTB) of ricin toxin contains two primordial galactose binding domains that are conserved among the large family of so-called R-type lectins (Dodd and Drickamer, 2001; Rutenber et al., 1987). That family of lectins includes the BoNT accessory protein HA1 (Inoue et al., 2003), as well as the galactose-/N-acetylgalactosamine (GalNAc)-binding lectin from the protozoan parasite *Entamoeba histolytica* (Frederick and Petri, 2005). Like RTB, HA1 and the protozoan GalNAc lectin are involved in promoting toxin/pathogen attachment to intestinal epithelial cells.

By lectin blot overlay and ELISA, we demonstrated that ricin recognizes the galactose-containing oligosaccharide side chains on human IgA1, IgA2, and SC (Mantis et al., 2004). Ricin attachment to the apical surfaces of T84 intestinal epithelial cell monolayers was reduced >10-fold by the addition of human colostrum SIgA, and this interaction was dependent on the immunoglobulin oligosaccharide side chains, not the antibody binding domain (Mantis et al., 2004). To examine whether this interaction could be relevant in vivo, we overlaid ricin onto tissue sections of human duodenum. The apical surfaces of enterocytes in vivo are coated with a 500-nm thick FBBG that is rich in glycoproteins, that we postulated may serve as a potential high avidity binding site for ricin (Frey et al., 1996; Mantis et al., 2000). Indeed, when applied to human intestinal tissue sections, ricin stained the luminal surfaces of intestinal villi strongly and uniformly (Fig. 2). The addition of molar excess amounts of SIgA reduced toxin binding to the intestinal villi in a dose-dependent manner. These data demonstrate that SIgA, in an FV-independent fashion, is sufficient to

block ricin, and probably other pathogen associated R-type lectins, from attaching to epithelial cell surfaces.

Interestingly, it has been proposed that SC itself, by virtue of its N- and O-linked carbohydrate side chains, also plays an important role in SIgA-mediated innate immunity in the intestine. Dallas and Rolfe, for example, demonstrated that purified SIgA in concentrations at or below those found in human milk inhibited the binding of *Clostridium difficile* toxin A to purified enterocyte brush border membrane receptors (Dallas and Rolfe, 1998). Toxin A bound to SC significantly more strongly than to it did to the heavy and light chains of IgA. Moreover, purified SC alone was sufficient to inhibit toxin binding to receptors. That initial report was recently confirmed and extended by Perrier and colleagues who identified the galactose and sialic acid residues on SC as being primarily responsible for blocking toxin A attachment to epithelial cell monolayers (Perrier et al., 2006). Furthermore, SC may serve as a decoy receptor for other pathogens, including enteropathogenic *E.coli* (Perrier et al., 2006).

### Immune exclusion

Immune exclusion is one of the most commonly proposed mechanisms by which SIgA blocks microbes from attaching to, colonizing and invading mucosal epithelia (Brandtzaeg, 2003; Kraehenbuhl and Neutra, 1992; Michetti et al., 1992; Michetti et al., 1994). In the context of the gut, immune exclusion is defined as the ability of SIgA, through its recognition of polyvalent antigens on the surfaces of viruses and bacteria, to agglutinate (*i.e.*, cross-link) microbial pathogens in the intestinal lumen and thereby retard or abrogate their ability to adhere to and/or penetrate the epithelium. Moreover, the mucus layer overlying the epithelium is proposed to not only limit diffusion of SIgA-immune aggregates, but to also actively entrap them and promote their clearance from the gut via peristalsis (Deplancke and Gaskins, 2001; Lievin-Le Moal and Servin, 2006) (Fig. 1).

The first report of immune exclusion is generally attributed to Williams and Gibson (Williams and Gibbons, 1972). Those investigators examined whether SIgA, through binding to and aggregation of bacteria, is sufficient by itself to prevent or reduce bacterial colonization on mucosal surfaces. In their experiments, *Streptococcus* species indigenous to the oral mucosa were mixed with SIgA purified from parotid saliva, and then examined by light microscopy for the ability to adhere to human buccal epithelial cells. It was observed that agglutinated bacteria were 3–4 times less capable of adhering to human epithelial cells than were mock-treated bacteria. While SIgA was used in these studies, the authors noted that this effect would probably occur with any class of antibody capable of promoting bacterial agglutination.

Nonetheless, subsequent studies have substantiated immune exclusion as a bona fide mechanism by which SIgA can protect the intestinal epithelium against infection. The most thoroughly documented example involves immunity to the enteropathogenic bacterium *Shigella flexneri* (Boullier et al., 2009; Phalipon et al., 2002; Phalipon et al., 1995). *S. flexneri* is the causative agent of shigellosis, or bacillary dysentery, a disease that is endemic in developing countries (Levine et al., 2007). *S. flexneri* infects the colonic mucosa, and like reovirus, first gains entry into the mucosa via M cells (Cossart and Sansonetti, 2004). To investigate the role of secretory antibodies in immunity to *S. flexneri*, Phalipon and colleagues produced a murine monoclonal dimeric IgA that was directed against the O-antigen of serotype 5a strain (Phalipon et al., 1994). The O-antigen, the serotype specific carbohydrate on the surface of *S. flexneri*, is known to be a primary target of antibodies in convalescent individuals (Ferreccio et al., 1991; Levine et al., 2007; Oberhelman et al., 1991; Phalipon et al., 1995; Van de Verg et al., 1990). The protective capacity of the anti-O antigen-specific monoclonal antibody, known as IgAC5, was demonstrated in a mouse

pulmonary infection model that mimics inflammatory lesions (*e.g.*, neutrophil influx) induced during natural intestinal infection (Phalipon et al., 1994). IgAC5 was protective against *S. flexneri* infection when instilled intranasally into mice 1 hr before bacterial challenge, or when administered passively using the so-called “backpack” tumor model (Kraehenbuhl and Neutra, 1992). Light microscopy, in conjunction with autoradiography, revealed that IgAC5 entraps *S. flexneri* within a thin layer of mucus overlying the respiratory epithelium (Phalipon et al., 2002). Similar results were obtained when comparable studies were performed in a rabbit ligated ileal loop model of shigellosis, demonstrating that immune exclusion occurs in the intestinal lumen, and not solely in the respiratory tract (Boullier et al., 2009). Another important facet of the above-described studies is the demonstration that IgAC5-mediated localization and protection against *S. flexneri* is dependent on SC. For example, IgAC5 in complex with recombinant human SC was ~10 fold more protective than was IgAC5 alone (Phalipon et al., 2002). Moreover, it was shown that the carbohydrate moieties on SC are not only responsible for enhancing IgAC5’s protective effects, but are also necessary for mucus localization (Phalipon et al., 2002).

Unfortunately, it is often assumed, based in part on early work by Stokes and colleagues (Stokes et al., 1975), that the simple association of SIgA with an epitope on the surface of a pathogen is sufficient to promote immune exclusion, and thereby confer protection *in vivo*. However, this is not always the case. In the case of reovirus, for example, IgA antibodies that bound the head region of the adhesion-like  $\sigma 1$  protein were protective, whereas antibodies against the viral capsid were not (Helander et al., 2004). These data reveal that coating reovirus with SIgA is itself not sufficient to confer protective immunity; rather SIgA must associate with specific functional epitopes on the viral surface. This has implications for vaccine design, especially against HIV-1. As noted by Kozlowski and Neutra, conventional neutralizing antibodies against HIV-1, as defined by their ability to block viral entry into CD4+ CCR5/CXCR4 T cells, are rare (Kozlowski and Neutra, 2003). It is therefore important to determine whether coating the virus with “non-neutralizing” antibodies are in fact sufficient to reduce (or prevent) transmission across mucosal surfaces. We and others have produced human monoclonal IgAs against surface expressed epitopes that will permit this to be tested in *in vitro* and/or *ex vivo* models of HIV-1 mucosal transmission (Mantis et al., 2007; Wallace et al., 2009; Wolbank et al., 2003).

### Direct effects of SIgA on bacterial virulence

Neither receptor blocking nor immune exclusion mechanisms can explain SIgA-mediated protection against a number of virulent enteropathogenic bacteria, such as *S. Typhimurium* and the closely-related *S. Enteritidis*. Like *S. flexneri*, *S. Typhimurium* initially infects the intestinal mucosa via Peyer’s patch M cells (Carter and Collins, 1974; Clark et al., 1994; Jones et al., 1994; Martinoli et al., 2007), although infection also occurs via villus enterocytes (Takeuchi, 1967; Vazquez-Torres et al., 1999). Contact with the epithelium is mediated by a number of fimbrial adhesins (Baumler et al., 1996a; Baumler et al., 1996b; Giannasca et al., 1996) and is augmented by bacterial motility (Marchetti et al., 2004). Motility of *S. Typhimurium* is driven by flagella, which are whip-like filaments (>10  $\mu\text{m}$  in length) that rotate coordinately to promote movement through viscous environments such as mucus (Berg, 2003). Once in contact with the intestinal surface, *S. Typhimurium* employs the so-called Salmonella pathogenicity island-1 (SPI-1) type three secretion system (T3SS) to invade Peyer’s patch M cells (Cossart and Sansonetti, 2004; Galan and Wolf-Watz, 2006). The SPI-1 T3SS is a syringe-like structure projecting from the bacterial surface that upon contact with host cells injects an array of bacterial proteins into the host cell cytosol, resulting in cytoskeletal rearrangements and bacterial macropinocytosis (Cossart and Sansonetti, 2004; Galan and Wolf-Watz, 2006). Strains of *S. Typhimurium* that lack certain

SPI-1-encoded effector proteins, or that are unable to display the SPI-1 T3SS on their surfaces, are unable to invade M cells and are avirulent by the oral route (Ellermeier et al., 2005; Galan, 2001; Jones et al., 1994). Infection of the intestinal mucosa by *S. Typhimurium* is associated also with an intense inflammatory response (McCormick, 2007; Raffatellu et al., 2008).

Following infection, the SIgA response to *S. Typhimurium* is directed almost exclusively against the O-antigen component of lipopolysaccharide (LPS) (Iankov et al., 2001; Iankov et al., 2002a; Michetti et al., 1992). In *S. Typhimurium*, the O-antigen consists of 20–35 repeats of tri- or tetrasaccharide units are anchored to the bacterial outer membrane via lipid A. It is estimated that more than 75% of the bacterial outer surface is covered with O-antigen (Nikaido, 1996). While the mucosal immune response following *Salmonella* infection is primarily directed against the O-antigen, the flagella are also a target of SIgA (Iankov et al., 2002a). Flagella-specific SIgA antibodies are primarily directed against flagellin (FliC), the protein that polymerizes to form the flagellar filaments.

It is well established that protection against *S. Typhimurium* and *S. Enteritidis* infection is conferred by SIgA antibodies directed against the O-antigen, but not those against the flagella (Carter and Collins, 1974; Iankov et al., 2004; Michetti et al., 1992). A role for O-antigen specific IgA in protective was demonstrated experimentally by Michetti and colleagues who produced a collection of dimeric monoclonal IgA antibodies directed against the *S. Typhimurium* O-antigen (Michetti et al., 1992). One of these monoclonal antibodies, Sal4, was characterized in detail (Michetti et al., 1992; Michetti et al., 1994). When delivered into the intestinal lumen by normal receptor-mediated transepithelial transport, Sal4 was sufficient to protect mice against a lethal oral challenge with *S. Typhimurium* (Michetti et al., 1992). Sal4 markedly reduced the numbers of bacteria that gained entry into the Peyer's patches following oral challenge, suggesting that the antibody interferes with bacterial attachment to and/or invasion of intestinal epithelial cells (Michetti et al., 1992). In agreement with the hypothesis, it was subsequently shown that the presence of Sal4 is sufficient to block invasion of polarized intestinal epithelial cell monolayers by *S. Typhimurium* in vitro (Michetti et al., 1994).

Similar findings were reported by Iankov and colleagues using *S. Enteritidis* as a model system (Iankov et al., 2004; Iankov et al., 2002b). Those investigators produced a collection of monoclonal IgAs against *S. Enteritidis* flagella (anti-H) and O-antigens (serotype O9) (Iankov et al., 2001; Iankov et al., 2002a). The O-antigen specific IgAs reduced *S. Enteritidis* invasion of HEp-2 intestinal epithelial cell monolayers, whereas the anti-flagellin antibodies did not (Iankov et al., 2002b). Similar outcomes were reported in vivo, in a model of generalized infection after intranasal inoculation of BALB/c mice. Specifically, the anti-O antigen monoclonal antibodies were protective, whereas the anti-flagellin antibodies were not (Iankov et al., 2004).

While both the above studies established that IgA antibodies against the O-antigen are protective against *Salmonella* infection, neither resolved the question of exactly *how* these antibodies interfered with the ability of the bacteria to invade intestinal epithelial cells. Nevertheless, a number of lines of evidence argue against a role for agglutination and, therefore, immune exclusion. For example, anti-flagella IgAs are more efficient than anti-O antigen specific antibodies at promoting *Salmonella* agglutination (a prerequisite for immune exclusion), yet flagella-specific IgAs are not protective in vitro or in vivo (Carter and Collins, 1974; Iankov et al., 2004; Iankov et al., 2002b). To directly address the role of agglutination in protection, we performed *S. Typhimurium* invasion assays in the presence of sub-agglutinating concentrations of Sal4 (Forbes et al., 2008). Under these conditions, Sal4 reduced the ability of *S. Typhimurium* to invade polarized epithelial cells monolayers



by ~20 fold. Sal4's protective effects were not augmented when the experiments were repeated under agglutinating conditions. Finally, as further evidence that agglutination is not integral to Sal4's protective effects, we performed invasion assays in the presence of O-antigen specific Fab fragments (Forbes et al., 2008). Monovalent Fabs, while unable to induce bacterial agglutination, blocked *S. Typhimurium* entry into epithelial cells almost as effectively as did Sal4. We conclude from these studies that agglutination, and by extension immune exclusion, cannot account for the effects of O-antigen specific IgA on Salmonella's entry into epithelial cells.

Based on the above data, we hypothesized that O-antigen specific IgA antibodies, specifically Sal4, must interfere with *S. Typhimurium*'s invasion of epithelial cells by another mechanism, possibility by one entailing bacterial motility and/or SPI-1 entry into epithelial cells. To investigate the impact of Sal4 on motility, we exposed *S. Typhimurium* to Sal4 at a range of concentrations and then monitored bacterial movement by video light microscopy. Digital video tracking analysis revealed that Sal4 induces a time- and dose-dependent arrest in bacterial motility (Forbes et al., 2008). Sal4's inhibitory effects were rapid (<15 min), and they were independent of the antibody's ability to promote bacterial agglutination. Moreover, Sal4-treated bacteria were completely "paralyzed" following antibody treatment. This is in contrast to what is observed when the bacteria are treated with anti-flagella (H) antibodies. In that case, the bacteria twitched and tumbled before ceasing to swim (Forbes et al., 2008). These experiments revealed that Sal4 is a potent inhibitor of bacteria motility, and that the mechanisms by which anti-flagella and anti-O antibodies alter *S. Typhimurium* motility are distinct.

Because non-motile mutants of *S. Typhimurium* are 30–50 fold less invasive than their parental wild type strains (Jones et al., 1992), we speculated that Sal4's effects on motility could account for the antibody's observed protective capacity. To examine this possibility, we performed bacterial invasion assays, in the absence and presence of Sal4, in which centrifugation was employed to artificially promote bacterium-host cell contact. To our surprise, Sal4-treated bacteria remained non-invasive, even though they adhered to the epithelial cell monolayers as effectively as untreated cells. These data reveals that Sal4 interferes with at least two distinct steps in the infection of epithelial cells by *S. Typhimurium*: it inhibits flagellum-based motility *and* blocks SPI-1 T3SS-dependent epithelial entry.

How might a Sal4 be interfering with both flagellum-based motility and SPI-1 T3S in *S. Typhimurium*? We hypothesized that Sal4, through its ability to associate with the O-antigen, perturbs the inherent organization of the bacterial LPS layer, and thereby destabilizes the integrity outer membrane. To investigate this possibility, we examined control- and Sal4-treated bacteria by scanning electron microscopy (SEM). Whereas the surfaces of control bacterial cells were uniformly grooved and ridged, the surfaces of Sal4-treated cells appear blistered (Fig. 3). The blisters (or blebs) were present on what were otherwise uncharacteristically smooth cell surfaces. IgAC5 had a similar effect on the surface of *S. flexneri* (S. Forbes and N. Mantis, manuscript in preparation). While the molecular basis of these changes remains unknown, it is clear that the association of IgA with LPS is sufficiently profound as to induce changes in the surface topology of the bacterial outer membrane and affect both motility and SPI-1 T3SS. These findings fundamentally challenge the previous assumptions as to how SIgA functions to combat microbial pathogens in the intestinal lumen (Fig. 4). Future studies in our laboratory will be aimed at understanding in molecular detail the sequence of events that occur at the bacterial cell surface following the association of Sal4 with the O-antigen.

## 4. Summary and perspective

In this review, we have summarized the current understanding of the three proposed mechanisms by which SIgA prevents pathogens from gaining access to the intestinal epithelium. The three mechanisms are (i) interference with epithelial receptor binding; (ii) immune exclusion; and (iii) direct impact on the activation or expression of virulence determinants (*e.g.*, motility and T3S). As these mechanisms are not necessarily mutually exclusive, it would not be surprising to discover that immunity against most viral and bacterial pathogens involves engagement of more than one of SIgA's activities. A more thorough understanding of SIgA's role in intestinal immunity will enable rational design of more effective mucosal vaccines, as well as the development more sophisticated surrogate markers that are necessary to evaluate these vaccines in human clinical trials (Levine, 2006).

## Acknowledgments

We gratefully acknowledge William Samsonoff of the Wadsworth Center's Electron Microscopy Core facility for assistance with the SEM. This work was supported in part by NIH grants HD061916 and AI082059 to NJM.

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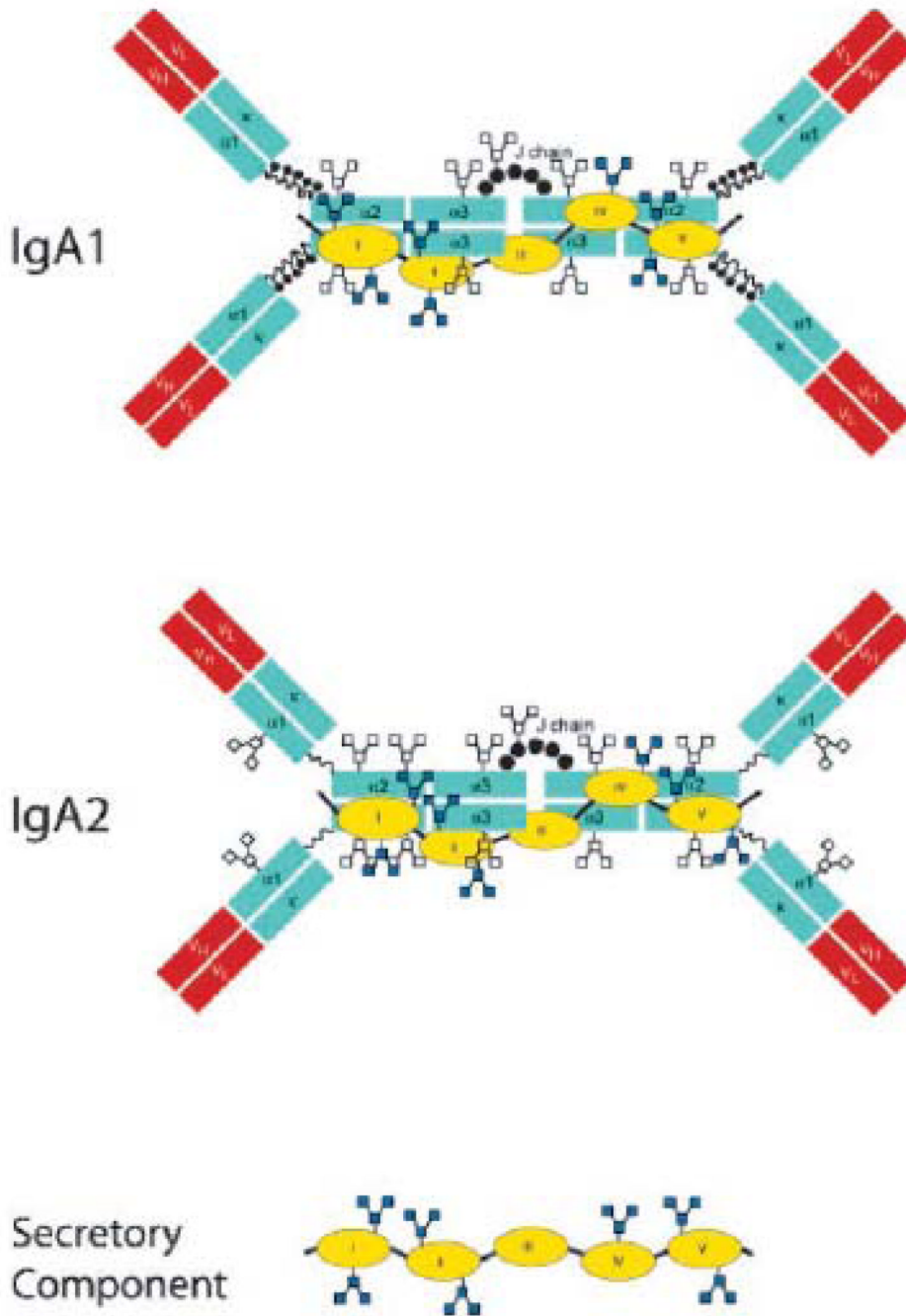
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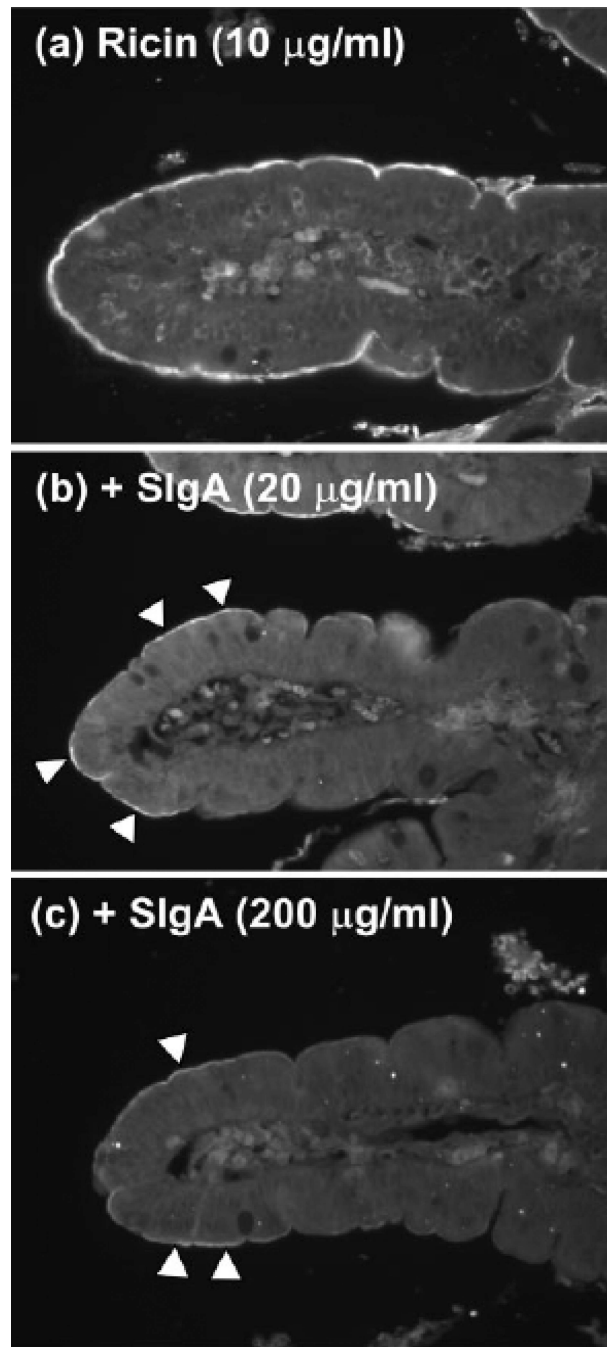
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**Figure 1. SIgA: Induction, transport and function in the intestinal lumen**

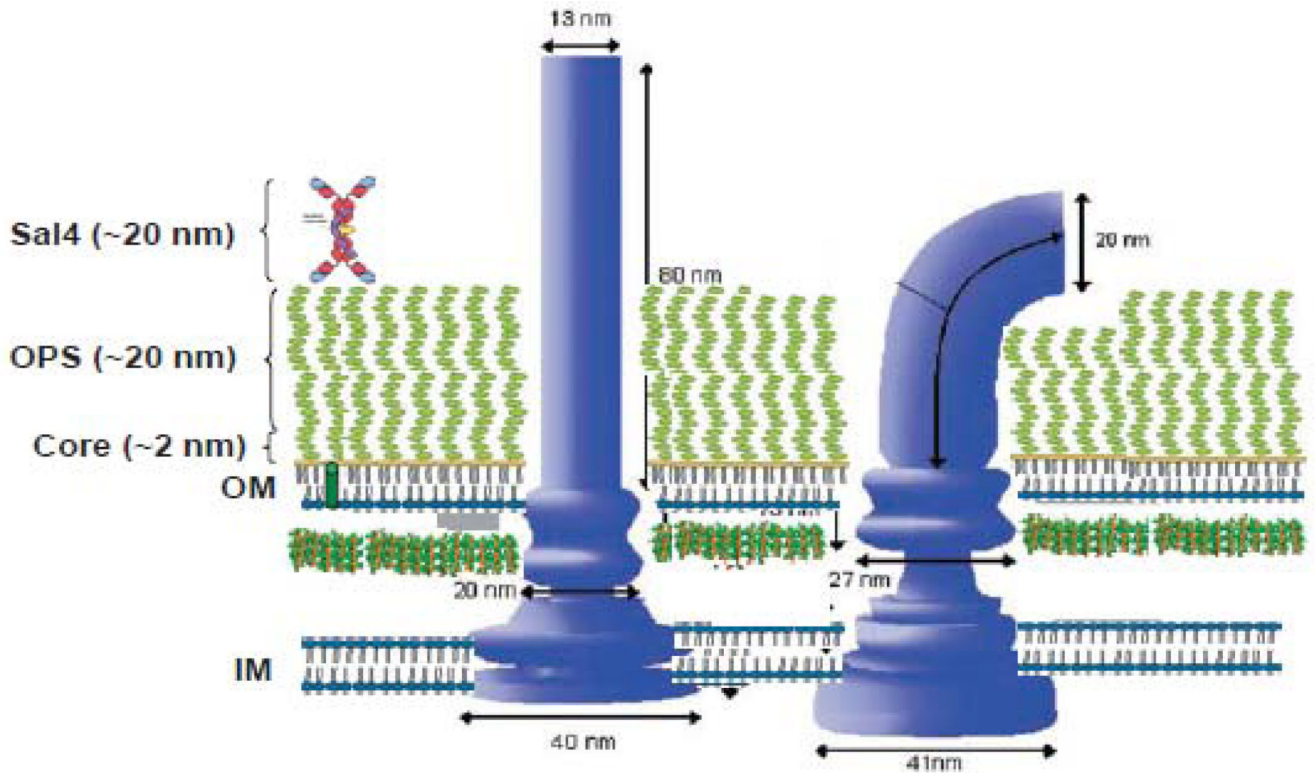
Particulate antigens (e.g., pathogens and toxins) present in the intestinal lumen are actively sampled by M cells present in the follicle-associated epithelium (FAE) overlying gut-associated lymphoid tissues (GALT). Following M cell transport, antigens are sampled by a network of sub-epithelial dendritic cells, which in turn present antigens to B cells within the regional lymphoid follicle. Following class-switch recombination and affinity maturation in the germinal center (dashed line), IgA-positive B cells emigrate from the GALT and seed the surrounding lamina propria. Dimeric IgA in the lamina propria is vectorially transported across the epithelium by the pIgR. Once in the intestinal lumen, SIgA can engage with cognate antigens on the surfaces of pathogens.



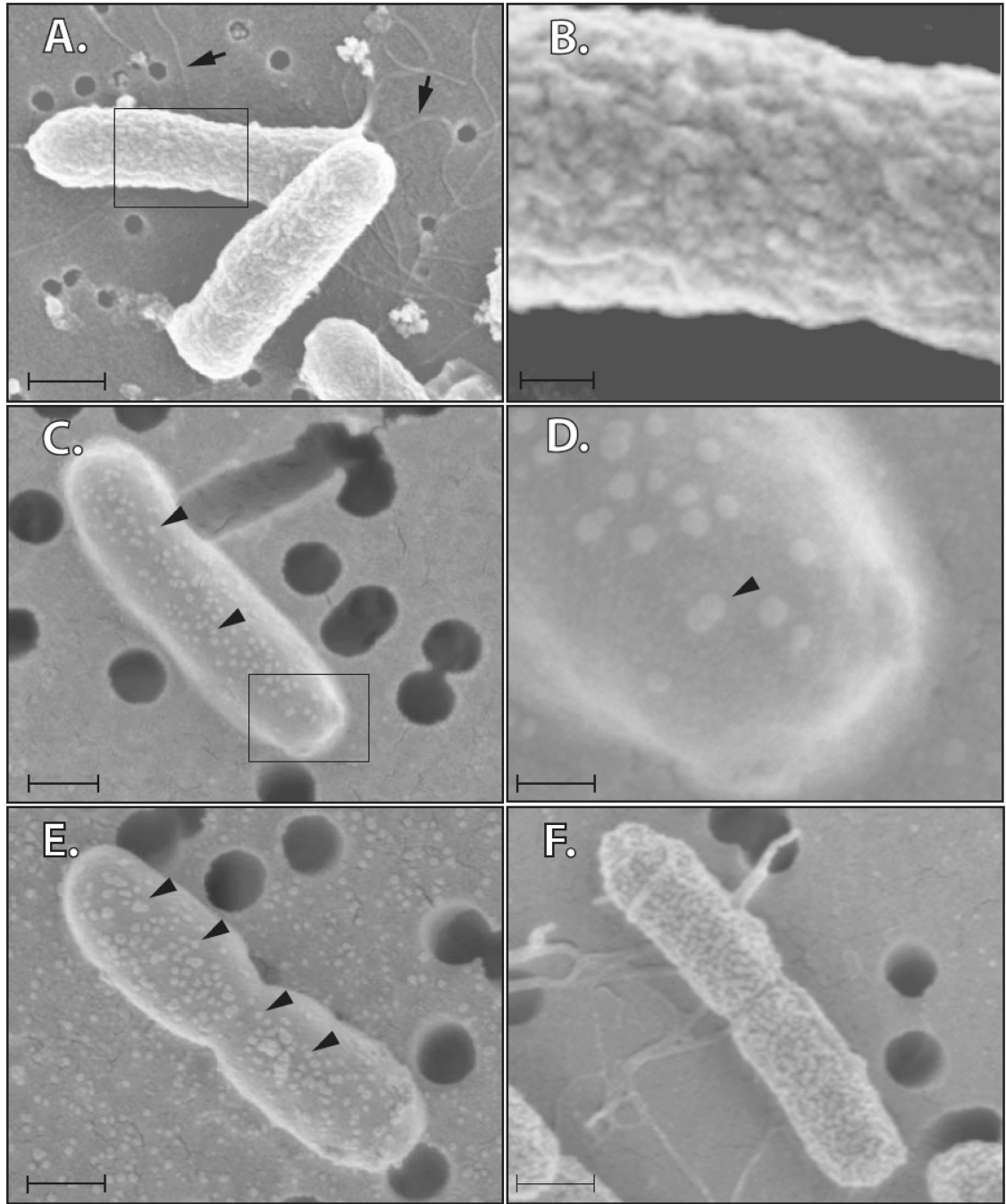
**Fig. 2. SIgA competitively inhibits ricin toxin binding to the luminal surfaces of human intestinal villi**

Biotinylated ricin (10 μg/ml) was incubated for 1 h with PBS (*a*) or indicated concentrations of sIgA (*b* and *c*), and then applied to deparaffinized tissue sections of human duodenum. Sections were then labeled with streptavidin-FITC and visualized by fluorescence microscopy. *a*, Ricin labeled the luminal aspects of intestinal villi strongly and uniformly. Ricin also weakly labeled cells within the lamina propria. *B* and *c*, Preincubation of ricin with indicated concentrations of sIgA Abs reduced toxin attachment to luminal aspects of the intestinal villi. Arrowheads highlight areas of visible toxin labeling. Figure reproduced

from (Mantis et al., 2004) with permission from the American Association of Immunologists.



**Figure 3. Sal4 induce changes in the surface topology of the outer membrane of *S. Typhimurium*** *S. Typhimurium* cells were incubated with TEPC-15 (a control monoclonal IgA of irrelevant antigen specificity), Sal4, anti-O or anti-H antibodies for 15 min, then captured on a porous (0.2 or 0.4  $\mu\text{m}$ ) filters, fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, dehydrated, critical-point dried, and then attached to an aluminum stud with carbon paste and sputter coated with gold. The specimens were observed at 5.00 kV using a LEO 1550VP FEG scanning electron microscope (Carl Zeiss SMT). **Panels A–B:** TEPC-15 (5  $\mu\text{g}/\text{ml}$ ); **Panels C–D:** Sal4 (5  $\mu\text{g}/\text{ml}$ ); **Panel E:** polyclonal anti-O antiserum (1:200 dilution); **Panel F:** polyclonal anti-flagella antiserum (1:200 dilution). Note flagella (Panel A, arrows). Membrane ‘blebs’ were observed on Sal4-treated cells (Panel C, arrowheads). In Panels A,C,E,F, the scale bars are equivalent to 0.5  $\mu\text{m}$ . In panels B,D the scale bars are 0.1  $\mu\text{m}$ . Polyclonal anti-sera were purchased from BD Difco (Franklin Lakes, NJ). The data presented in this figure are part of a manuscript in preparation by the authors of this review.



**Figure 4. Proposed mechanisms by which O-antigen specific SIgA protects against *S. Typhimurium* infection**

(1) *S. Typhimurium* proliferates within the intestinal lumen and propels itself via flagella-based motility; (2) Upon contact with intestinal epithelial cells, the bacteria promote their own uptake and engulfment. This process is mediated by the SPI-1 T3S system; (3) Agglutination of *S. Typhimurium* in the presence of LPS specific SIgA antibodies, such as Sal4. This process would occur only at optimal antibody-bacteria ratios; (4) An alternative model by which Sal4 may arrest *S. Typhimurium*. In this case, the association of Sal4 with the bacterial LPS inhibits flagella-based motility, reduces SPI-1 T3S activity, and induces

alterations in the bacterial outer membrane (depicted as a halo surrounding the bacterial cells).

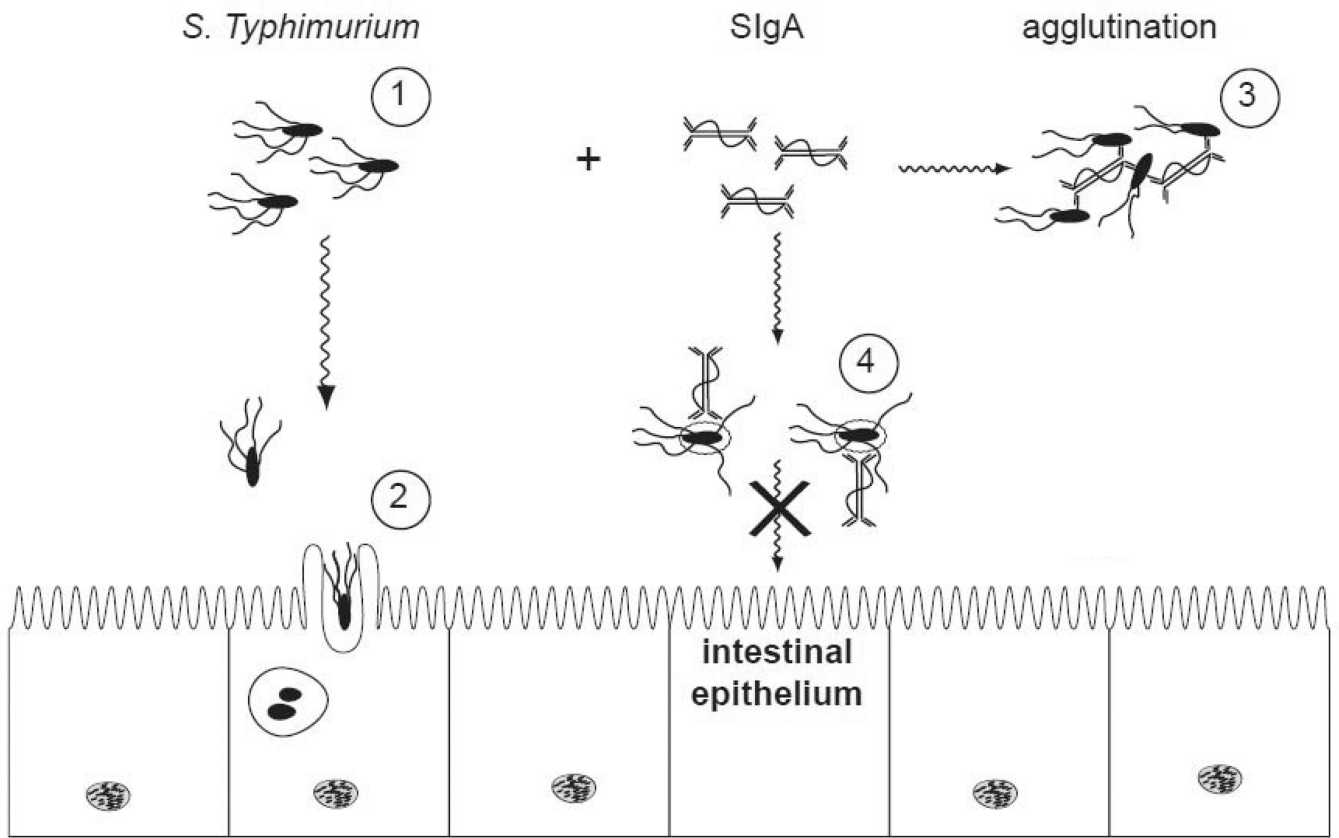


Fig 5.