Secretory IgA-Mediated Neutralization of Shigella flexneri Prevents Intestinal Tissue Destruction by Down-Regulating Inflammatory Circuits

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Secretory IgA-Mediated Neutralization of *Shigella flexneri* Prevents Intestinal Tissue Destruction by Down-Regulating Inflammatory Circuits

Séverine Boullier,*† Myriam Tanguy,‡§ Khalil A. Kadaoui,¶ Cécile Caubet,*† Philippe Sansonetti,‡§ Blaise Corthesy,¶ and Armelle Phalipon2‡§

*Shigella*, a Gram-negative invasive enteropathogenic bacterium responsible for bacillary dysentery, causes the rupture, invasion, and inflammatory destruction of the human colonic mucosa. We explored the mechanisms of protection mediated by *Shigella* LPS-specific secretory IgA (SlgA), the major mucosal Ab induced upon natural infection. Bacteria, SlgA, or SlgA-S. *flexneri* immune complexes were administered into rabbit ligated intestinal loops containing a Peyer’s patch. After 8 h, localizations of bacteria, SlgA, and SlgA-S. *flexneri* immune complexes were examined by immunohistochemistry and confocal microscopy imaging. We found that anti-*Shigella* LPS SlgA, mainly via immune exclusion, prevented *Shigella*-induced inflammation responsible for the destruction of the intestinal barrier. Besides this luminal trapping, a small proportion of SlgA-S. *flexneri* immune complexes were shown to enter the rabbit Peyer’s patch and were internalized by dendritic cells of the subepithelial dome region. Local inflammatory status was analyzed by quantitative RT-PCR using newly designed primers for rabbit pro- and anti-inflammatory mediator genes. In Peyer’s patches exposed to immune complexes, limited up-regulation of the expression of proinflammatory genes, including TNF-α, IL-6, Cox-2, and IFN-γ, was observed, consistent with preserved morphology. In contrast, in Peyer’s patches exposed to *Shigella* alone, high expression of the same mediators was measured, indicating that neutralizing SlgA dampens the proinflammatory properties of *Shigella*. These results show that in the form of immune complexes, SlgA guarantees both immune exclusion and neutralization of translocated bacteria, thus preserving the intestinal barrier integrity by preventing bacterial-induced inflammation. These findings add to the multiple facets of the noninflammatory properties of SlgA. *The Journal of Immunology*, 2009, 183: 5879–5885.

A

t important activity of mucosal surfaces is the production of Abs referred as to secretory IgA (SlgA)3 that serves as the first line of defense against pathogenic microorganisms (1, 2). SlgA also limits the penetration of commensal bacteria through the epithelium and contributes to shape the density of different bacterial species in the intestinal lumen (3, 4). Mucosal SlgA Ab, a remarkably stable molecule (5, 6), exists in dimeric and polymeric forms (pIgA) comprising the joining chain (J chain protein) added during Ab secretion from mucosally acti-

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3 Abbreviations used in this paper: SlgA, secretory IgA; DC, dendritic cell; EC, epithelial cell; HPRT, hypoxanthine phosphoribosyltransferase; hSC, human SC; LB, Luria-Bertani; pIgA, polymeric IgA; pIgR, polymeric Ig receptor; PP, Peyer’s patch; SC, secretory component; SED, subepithelial dome; TTSS, type III secretion system.

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of the inflammatory process (24, 25). In such a model, both anti-LPS SIgA and IgG were shown to protect the host from tissue destruction resulting from *Shigella*-induced inflammation (10, 25, 26). In vitro studies using human intestinal crypt cells have also revealed that anti-LPS plgA and bacterial LPS colocalize in the apical recycling endosome, impairing LPS-induced NF-κB translocation and subsequent proinflammatory responses (15).

The aim of the current work was to extend our understanding of anti-*Shigella* LPS SIgA-mediated mechanisms of protection in vivo at the intestinal level. *Shigella* alone or in combination with a specific anti-LPS SIgA were previously used to unravel the capacity of SIgA to mediate Ag transport across PPs in the mouse (19). However, because mice are resistant to intestinal infection with *Shigella*, the model is not fully appropriate to address the anti-inflammatory properties of SIgA in mucosal tissues. Hence, in the current study, we used the rabbit model of ligated ileal loops infected with *Shigella* because it mimics both local inflammation and subsequent massive tissue destruction as it occurs during natural infection in humans (24, 27). Although this model has intrinsic limitations due to the restricted number of available immunologic reagents, it turned out to be very helpful to demonstrate that specific SIgA is a key player to reduce the proinflammatory response turned on by *Shigella* at the level of the intestinal barrier in vivo.

Materials and Methods

**Protein production and purification**

Hybridoma cells producing IgAC5 specific for *S. flexneri* serotype 5a LPS (10) were cultured in complete RPMI 1640 medium as described (28). High-density cell cultures were conducted in Celline-350 bioreactors (Integra Biosciences). Supernatants were harvested twice a week, filtered through 0.22-μm filters and concentrated using a Centricon-30 YM30 centrifugal filter device (Millipore). SIgA molecules were obtained by combining pIgA molecules with hSC in recombinant human SC (hSC) prepared as previously described (10). Abs were purified through multiple chromatographic steps (28). Purified recombinant human SC (hSC) was prepared as previously reported (10). SIgA molecules were obtained by combining pIgA molecules with hSC in PBS according to the conditions described in Crottet and Courtésy (6). Cy3-SIgA molecules were obtained by conjugation to Cy3 using the FluoroLink mAb Cy3 labeling kit (Amersham Biosciences) according to the procedure provided by the manufacturer. Radiolabeled SIgA was obtained as previously described (10). To visualize its localization, 10^6 cpm were added with the immune complexes, one or two with SIgA and bacterial LPS, followed by incubation for 90 min at 4°C in PBS containing 12% sucrose (Merek), followed by an overnight incubation at 4°C in PBS containing 18% sucrose. For final embedding, ileal loop portions were immersed in optimum cutting temperature reagent (Sakura Finetek). The tissue was then snap-frozen in isopentane (Sigma-Aldrich) cooled in liquid nitrogen. Frozen sections were generated using a cryostat (Leica Microsystems) and used immediately or kept at −20°C in the dark.

**Immunolabeling of rabbit DCs**

Confocal microscopy pictures were obtained using a Leica TCS NT microscope (Leica Microsystems). Excitation was obtained with an argon-krypton laser, with lines set at 488, 568, and 647 nm for FITC, Cy3, and Cy5 fluorochromes, respectively. Images were taken with a 20× or 63× objective and processed using the Leica TCS NT software (Leica Microsystems).

**Analysis of cytokine expression by quantitative real-time RT-PCR**

Total cytoplasmic RNA was extracted from freshly isolated PP cells using TRIzol (Invitrogen) and RNeasy spin columns (Qiagen), according to the manufacturer’s instructions. All RNA samples were treated with on-column RNase free DNase I (Qiagen). Total RNA (100 ng) was converted into cDNA with Superscript II reverse transcriptase (Invitrogen). The nucleic acids were stored at −70°C until assayed by real time RT-PCR. Oligonucleotide primers for Cox-2, TNF-α, IL-6, IFN-γ, IL-10, and the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) were chosen using the *Oryctolagus cuniculus* cDNA sequences available in GenBank (http://www.ncbi.nlm.nih.gov/Entrez/index.html), and designed using the primer3 software (http://frodo.wi.mit.edu/primer3). Primer sequences were checked with the BLAST (basic local alignment search tool) software (http://www.ncbi.nlm.nih.gov/BLAST) to prevent homologies to undesired genes and other coding sequences. Oligonucleotide primers were synthesized by Eurogentec. The primer sequences and expected PCR product length are shown in Table I. For each gene, a standard curve was established using serial dilutions of an external standard, consisting of the sequence of target genes inserted into the PCR XL-TOPO plasmid (Invitrogen). From the calculated copy number of double-stranded plasmid DNA, serial dilutions (1/10) of known input copy numbers (10^7 to 10^10) for each target gene were used to generate the standard curves. All
amplifications of standard mixes were performed in duplicates that were included on each PCR plate to guarantee quantitation of the tested gene.

The real-time RT-PCR mixture contained 5 µl of sample cDNA (1/10 dilution of the reverse transcriptase reaction in DNase/RNase-free water) and 20 µl of a mix consisting of 2.5 µl of forward and reverse primers (300 µM each), 2.5 µl of tRNA (3 ng/µl), and 12.5 µl of the iTaq SYBR Green Supermix (Bio-Rad). Each sample was tested in triplicate for every couple of primers. Reactions were run on an ABI Prism sequence detection system 5700 instrument (Applied Biosystems). PCR conditions were 40 cycles at 95°C for 15 s and 60°C for 1 min. A post-PCR dissociation curve was run to assess the specificity of the amplified sequences. Results were analyzed with the ABI sequence detection system software (Applied Biosystems). The PCR threshold cycle number for each sample was calculated at the point where the fluorescence signal exceeded the threshold limit, fixed at 10 to 20 SDs above the average background fluorescence. From the threshold cycle number and the standard curves, the number of gene copies was calculated. Normalization of the results was performed using primers targeting HPRT expression. Statistical analysis of the data was done using primers targeting HPRT expression. Statistical analysis of the data was done using the Wilcoxon signed-ranks test. Differences between groups were considered significant when \( p \) values <0.05 were obtained.

Results

Shigella-specific SIgAC5 prevents bacteria-induced destruction of the intestinal barrier

Intraluminal infection of rabbit ligated ileal loops with Shigella drives the induction of a major destructive inflammatory response (30), mimicking that observed in rectal biopsies of Shigella-infected patients upon natural infection (32). It thus represents an accurate model to examine at the intestinal level the cellular and molecular consequences of bacterial neutralization mediated by SIgAC5, a monoclonal dimeric IgA specific for S. flexneri serotype 5a LPS O-Ag (25). After administration of the bacteria into ileal loops, massive loss of the architecture of PPs was observed as a consequence of the proinflammatory phenotype of Shigella (Fig. 1A). Elsewhere in the intestine, tissue damages were reflected by effacement of the villus structure (Fig. 1B). In contrast, passive administration of agglutinating amounts of SIgAC5, together with a pathology-inducing amount of bacteria, resulted in the maintenance of the integrity of the intestinal barrier at the level of both PPs and villi during the 8-h incubation (Figs. 1, C and D). These results suggest that the protective efficacy of Shigella-specific SIgAC5 in the intestinal environment is due to immune exclusion. Moreover, these data suggest that SIgAC5-bacteria immune complexes do not dissociate in the harsh intestinal environment during the 8-h incubation (shown later in Fig. 3C), thus supporting the adequacy of the model to investigate the nature of other molecular mechanisms of SIgA-mediated mucosal homeostasis operating, in addition to immune exclusion.

FIGURE 1. SIgAC5 prevents Shigella-induced destruction of the intestinal barrier. Histologic examination of intestinal tissue sections obtained from rabbit ileal loops 8 h postadministration with a single dose of either 10⁹ Shigella alone (A and B) or combined to agglutinating amounts of SIgAC5 (C and D). Following paraffin embedding, H&E staining was performed. Exposure to Shigella triggers inflammation accompanied with destruction of the architecture of PP (compare A and B) and of the epithelial barrier with shortening of the villi length (compare B and D). Typical examples of results of histological analysis of more than five samples are shown. L, Lumen. Magnification, ×100 for A, B, and D and ×40 for C.

### Table 1. Primer sequences for rabbit cytokines, Cox-2, IcsA, and HPRT for standard plasmids^a^

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<th>Sequence (5′–3′)</th>
<th>Tm (°C)</th>
<th>Amplicon Length (bp)</th>
<th>Accession No.</th>
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</table>

^a^ bp. Base pairs; F, forward, R, reverse.

we analyzed the localization of both SIgAC5 and compartment Shigella-specific SIgAC5 preferentially associates with mucus in loops 8 h postadministration with either Shigella alone, radiolabeled SIgAC5 alone, or a combination of the bacterium and agglutinating amounts of SIgAC5 were analyzed. Treatment with photographic emulsion shows the luminal, mucus-associated localization of radiolabeled SIgAC5, when administered alone (A, arrows) or combined with Shigella (B, arrows). C. Confirmation of the presence of abundant mucus trapping the Ab is depicted after staining with Alcian blue of the section adjacent to that of the picture presented in A. D. Bacterial staining (in brown) using an anti-S. flexneri 5a LPS polyclonal serum is limited to the epithelial surface in sections of ileal loops administered Shigella-SigAC5 immune complexes. In contrast, a largely diffuse bacterial labeling revealing massive invasion of the mucosa is observed when the bacterium is delivered alone (E). Examples of typical results of histological analysis of more than five samples are shown. L, Lumen. Magnification, ×100 for all panels.

Shigella-specific SIgAC5 preferentially associates with mucus in the intestinal lumen and restricts the bacteria in this compartment

To demonstrate that immune exclusion was indeed taking place, we analyzed the localization of both SIgAC5 and Shigella, alone or in combination, when administered into the rabbit ligated ileal loop. In the mouse respiratory tract, anchoring of SIgA within the mucus via SC was shown to be essential to the function of immune exclusion of the Ab (10). Similarly, we found that radiolabeled SIgAC5 accumulated as clusters in the rabbit intestinal lumen when administered alone (Fig. 2A). Using 5 μm-adjacent histological sections stained with the mucus dye Alcian blue, distribution of radiolabeled SIgAC5 was shown to be limited to areas rich in mucus (Fig. 2C). Consistent with the local absence of mucus, the Ab molecule was barely detectable in the neighborhood of the surface of PPs (data not shown). Immune complexes were also found as clusters (Fig. 2B) and in association with mucus within the lumen (data not shown). In support of the selective localization of SIgA, detection of Shigella with a specific anti-LPS antisera showed a predominant distribution of the bacteria neutralized by SIgAC5 next to the EC surface (Fig. 2D), in contrast to its wide tissue dissemination when administered alone (Fig. 2E). Similar to the lung tissue (10), these results verify that SIgA binding to mucus is a key feature to ensure optimal immune exclusion in the intestine as well.

SIgAC5-Shigella immune complexes are translocated into PPs and colocalized with DCs in the subepithelial dome (SED) region

In mice, SIgA Abs have been shown to enter PPs, then targeting DCs in the SED region (17). In addition, specific SIgA have been reported to promote bacterial translocation into mouse PPs in the form of immune complexes (19). Therefore, we first analyzed whether in the rabbit model highly sensitive to S. flexneri, SIgA-S.

Shigella, S. flexneri complexes were also translocated into PPs and whether this impacted on the tissue wellness. As shown in Fig. 3A, moderate bacterial labeling using a specific anti-LPS antisera was observed in the SED region of the PPs exclusively, in comparison with the pronounced accumulation of bacteria-SIgA complexes on the surface of EC (Fig. 2D). Real-time RT-PCR, using expression of the Shigella-specific gene icsA, which encodes the virulence effector responsible for bacterial spreading within tissues (33), was performed to quantify translocated Shigella. Expression of icsA transcripts was found in the PPs of loops administered with the immune complexes (Fig. 3B, open symbols), providing evidence that under these experimental conditions, Shigella was no longer
able to damage the neighboring tissues. The level of transcripts was ~300 times lower than that found in the PPs associated with ileal loops administered Shigella alone (Fig. 3B, filled symbols), most likely reflecting Ab-mediated blocking of translocation, rather than inhibition of icsA gene expression upon IgA binding.

To further verify whether Shigella was associated with SIgAC5 in this compartment, colocalization of red Cy3-SIgA and GFP: Shigella was analyzed by confocal microscopy imaging. Stable immune complexes were indeed readily detected in the SED compartment of PPs, as reflected by the appearance of numerous yellow spots (Fig. 3C, arrowheads). After uptake from the intestinal lumen, we observed colocalization of blue Cy5-labeled CD11c low spots (Fig. 3C). In many instances, turquoise-green spots resulting from the superimposition of yellow SIgAC5-Shigella immune complexes and blue Cy5-labeled DCs were observed throughout the SED region. Detectable, although more diffuse, turquoise fluorescence coloring the cytoplasm of a selection of DCs was additionally showing up, suggesting that GFP: Shigella was indeed internalized, leading to release of bacteria-expressed cytoplasmic GFP.

**Binding to SIgAC5 restricts the Shigella-induced inflammatory response in PPs, thus preserving their integrity**

Because no destruction of the PPs was observed (Fig. 1C) despite the presence of Shigella in complex with SIgAC5 in the SED region (Fig. 3, A and C), we made the assumption that the proinflammatory pathways usually induced by Shigella were quenched when the bacterium was bound to SIgAC5. To test this hypothesis, we measured the expression of a selection of genes encoding pro- and anti-inflammatory cytokines and mediators. As protein-specific reagents are missing for the rabbit species, we designed a battery of oligonucleotide primers to track, by quantitative real-time PCR, the expression of IL-6, IL-10, TNF-α, IFN-γ, and cyclo-oxygenase-2 (cox2) genes (Table II). In comparison with control cells recovered from a PP exposed to SIgA alone, administration of Shigella within the ileal loop led to a strong increase of the expression of all tested proinflammatory mediators within cells isolated from PPs (ranging between 10- to 100-fold, depending on the mediator). We found that the same set of mediators were also expressed in the PP cells associated with ileal loops administered with the immune complexes, yet to a statistically significant lesser extent (Fig. 4), that interestingly turned out to be appropriate to preserve tissue organization. Reduction of the expression of proinflammatory mediators by cells recovered from PPs exposed to immune complexes was not accompanied by the expected increase of IL-10 known to contribute to intestinal homeostasis (Fig. 4). As a matter of fact, expression of IL-10 remained equivalent in the various experimental settings assayed, and appears thus as a “default” status, which is overcome in inflammatory situations. This result suggests that SIgA Abs exert an anti-inflammatory effect in the PPs, which relies more on a reduction of the proinflammatory responses rather than on the rapid induction of anti-inflammatory pathways.

**Discussion**

In this study, we report that both immune exclusion and SIgA-mediated translocation of Shigella into PPs aids the preservation of the intestinal barrier integrity by preventing bacteria-induced inflammation. These data constitute the first demonstration that SIgA specific for Shigella LPS are protective in the intestinal environment.

Because SC is crucial for SIgA protective functions (10), we used the optimal and natural molecular form of the Ab, i.e., SIgA, in the rabbit ileal loop model which mimics the intestinal inflammation induced by Shigella upon natural infection in humans. However, although 100 to 1000 bacteria appear to be sufficient in humans to trigger the disease (23), 109 bacteria are required in the rabbit ileal loop model which mimics the intestinal inflammation induced by Shigella upon natural infection in humans.
Interestingly, we observed that the translocation of SlgAC5-Shigella immune complexes did not induce PP destruction. Preservation of PP integrity can be explained by the limited up-regulation of the expression of proinflammatory cytokines in the cells isolated from PPs of ileal loops administered SlgAC5-Shigella as compared with Shigella alone. We speculate that two plausible mechanisms underlying this protective effect are likely to occur. On one hand, reducing the inflammatory response in PP might be the consequence of SlgA-Shigella immune complexes targeting tolerogenic CD11c+ CD11b+ DCs (19), with the subsequent modulation of the host immune response toward anti-inflammatory pathways as previously reported (4, 18, 38). The lack of appropriate anti-rabbit CD11b mAb prevented us from experimentally confirming this hypothesis. The observation that the level of homeostatic IL-10 is slightly affected during inflammation suggests that the default tolerogenic status of the epithelium dominates as long as pathogenic stimuli do not instruct the local immune system to activate proinflammatory circuits. On the other hand, anti-LPS SlgA bound to Shigella might act by blocking pathways of the bacterial virulence program. Indeed, Shigella uses a type III secretion system (TTSS) to inject virulence effectors into human cells, leading to bacterial invasion and a vigorous inflammatory response. Previous results have shown that the TTSS efficiency depends on the length of the LPS chains at the bacterial surface. Strains with truncated LPS are highly proficient at invading cells in vitro, possibly through enhanced access of the TTSS to host cells, whereas the extended LPS isofrom impairs TTSS function, which leads to reduced virulence within the gastrointestinal tract (39). Thus, by binding to LPS chains at the bacterial surface and promoting bacterial agglutination, anti-LPS SlgA is likely to impair the accessibility of the TTSS to host cells.

The current study increases our understanding of the mechanisms mediated by anti-LPS SlgA in protection against Shigella infection. We now know that, in addition to immune exclusion (Ref. 10 and the current work) and intracellular neutralization of Shigella LPS by IgAC5 during pIgR-mediated transport of the Ab molecule in EC (15), translocation of a small proportion of SlgA-based immune complexes into PP contributes to downregulate proinflammatory responses. This further emphasizes the need to develop adequate strategies to prime mucosal immune responses, including SlgA-mediated Ab response, upon vaccination against enteric pathogens, as it appears from our data that the Ab is involved at different stages in the control of the mucosal homeostasis.

Disclosures
The authors have no financial conflict of interest.

References


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