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Hemoglobin Transforms Anti-Inflammatory *Salmonella typhi* Virulence Polysaccharide into a TLR-2 Agonist

Rohini Garg and Ayub Qadri

Vi capsular polysaccharide is a major virulence determinant of the human typhoid-causing pathogen *Salmonella typhi*; it is absent in nontyphoidal *Salmonella* serovars. We show in this study that through its specific interaction with the membrane recognition complex containing the prohibitin family of molecules, Vi can inhibit the production of inflammatory cytokines from mononuclear phagocytes stimulated with *Salmonella* flagellin. Remarkably, Vi lost this anti-inflammatory capability and switched to a proinflammatory state when cell stimulations were performed in the presence of serum. The serum-transformed proinflammatory form of Vi induced secretion of cytokines from monocytes by specifically engaging TLR-2/TLR-1. The molecule responsible for bringing about this conversion of Vi from an anti-inflammatory to a proinflammatory form was serum-derived hemoglobin. Derivatives of Vi incapable of interacting with hemoglobin did not switch to a proinflammatory state in vitro or in vivo. These findings provide compelling evidence for a role of hemoglobin in transforming the anti-inflammatory *S. typhi* virulence polysaccharide into an immune activator. *The Journal of Immunology*, 2010, 184: 5980–5987.

Microbial pathogens are sensed by the host immune system through germline encoded pattern recognition receptors (PRRs) including TLRs, nucleotide binding and oligomerization domain-like receptors (NLRs) and lecins (1). These receptors recognize conserved pathogen-associated molecular patterns that include lipids, polysaccharides, proteins, and nucleic acids (1). In addition to these cell membrane-associated and cytosolic sensors, several circulating host factors have been shown to enhance inflammatory responses produced by microbial components (2–4). Collectively, the responses produced through engagement of PRRs contribute to inflammation and constitute an important component of host defense against a large number of pathogens (5, 6). Many pathogens have devised ways to counter these protective responses by interfering with intracellular signaling events transduced through PRRs. This interference is achieved either through engagement of inhibitory receptors at the membrane or through intracellular delivery of inhibitory molecules (7–9).

Vi is a linear polymer of 1,4(2-deoxy)-2-N-acetylgalacturonic acid variably O-acetylated at the C3 position (10–12). It constitutes a major distinction between *Salmonella typhi*, which produces typhoid almost exclusively in humans, and nontyphoidal serovars, such as *Salmonella typhimurium*. The latter causes only self-limiting gastroenteritis in humans. Vi protects *S. typhi* from the action of anti-O Ab and renders it resistant to phagocytosis and complement-mediated killing (13). Vi also enhances survival of *S. typhi* in cultured macrophages (14). Typhoid rates are significantly higher in volunteers infected with capsulated serotype Typhi strains than in those infected with passaged derivatives lacking the Vi Ag. Although noncapsulated serotype Typhi strains can still cause typhoid fever, in vivo data suggest that the loss of Vi results in considerable attenuation (15). Abs to Vi protect against *S. typhi* infection and Vi is currently in use as a vaccine against typhoid in humans (16–19). Recently, we have shown that this polysaccharide could downregulate early chemokine secretion from intestinal epithelial cells (IECs) during infection with *S. typhi* by targeting the prohibitin family of molecules. This downregulation was associated with reduced activation of ERK (7).

In the current study, we show that Vi can also inhibit TLR-5-induced inflammatory responses from mononuclear phagocytes under serum-free conditions by targeting membrane-associated prohibitin and BAP-37. Serum completely abrogated these anti-inflammatory effects and converted Vi into a TLR-2 agonist. The molecule in serum that suppressed the immune-inhibitory capability of Vi and produced a proinflammatory species from it was found to be hemoglobin. These results assign a novel role to hemoglobin in thwarting anti-immune capability of typhoid virulence polysaccharide and bringing about its switch to a proinflammatory state.

**Materials and Methods**

**Cells and reagents**

The human monocytic cell lines THP-1 and U937 were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 supplemented with 10% FCS at 37°C in a humidified CO2 (5%) incubator. The LPS-hyporesponsive bone marrow-derived mouse macrophage cell line, GG2EE, was provided by Dr. Steven B. Mizel (Wake Forest University, Winston-Salem, NC). HEK-293T was maintained in DMEM supplemented with 10% FCS and BAP-37. Serum completely abrogated these anti-inflammatory effects and converted Vi into a TLR-2 agonist. The molecule in serum that suppressed the immune-inhibitory capability of Vi and produced a proinflammatory species from it was found to be hemoglobin. These results assign a novel role to hemoglobin in thwarting anti-immune capability of typhoid virulence polysaccharide and bringing about its switch to a proinflammatory state.
obtained from Sigma-Aldrich. Abs specific to prohibitin and BAP-37 were prepared by immunizing rabbits with C-terminal peptides, which are different between prohibitin and BAP-37 (20). Animal experiments were performed according to the guidelines provided by the Institutional Animal Ethics Committee of the National Institute of Immunology.

Cell stimulation and cytokine analysis

U937 and THP-1 cells were incubated with flagellin [isolated from S. typhimurium as described by Smith et al. (21)] in the presence or absence of Vi for 6 h at 37°C in a 96-well plate in triplicate. Stimulations were performed in RPMI 1640 supplemented with or without 10% FCS. THP-1 cells were activated with Vi in the presence of serum for 6 h (for IL-8) and for 24 h (for TNF-α). For IL-6, THP-1 cells were first activated with PMA (100 ng/ml) for 24 h and then incubated with Vi for an additional 24 h in the presence of serum. Cytokines were analyzed by commercially available ELISA (Opt EIA; BD Pharmingen, San Diego, CA). Human PBMCs or immature murine bone marrow-derived dendritic cells (BMDCs) were also stimulated with flagellin in the absence or presence of Vi, and supernatants were assayed for IL-8, TNF-α, and IL-6 by ELISA.

Binding of Vi to cells and immunoprecipitation

The binding of Vi to U937 and THP-1 cells was analyzed by flow cytometry, and Vi-interacting molecules in U937 cells were identified by immunoprecipitation as described earlier (7) with slight modifications. Cells (2 × 10⁶) were washed and incubated with Vi (1 μg per 10⁶ cells) for 1 h at 4°C. Subsequently, cells were washed with PBS and lysed in TKM lysis buffer (Tris HCl 50 mM [pH 7.4], KCl 25 mM, MgCl₂ 5 mM, EDTA 1 mM, nitrocellulose (NC) membrane and blotted with rabbit anti-BAP-37 Ab, acrylamide gel. The immunoprecipitated proteins were transferred to an Laemmli sample buffer (nonreducing) and run in a 12% SDS polyacrylamide gel. The immunoprecipitated proteins were transferred to a polyvinylidene difluoride membrane, and proteins were transferred to an NC membrane. The presence of Vi in different fractions was visualized by bloting the membrane with Abs to Vi.

Prohibitin knockdown

To obtain stable repression of prohibitin protein, 60-base-long oligonucleotides (containing a unique 19-nt sequence targeting coding regions of prohibitin) were cloned into pSUPER (neo+gfp) expression vector system (Oligoengine, Seattle, WA). The resulting transcript from recombinant vector forms short hairpin RNA (shRNA) that is cleaved in cells showing that siRNAs against prohibitin were target-specific (Fig. 3C). The possibility that Vi might be pulling down mitochondrial prohibitin was ruled out even when the polysaccharide was added to cells after stimulation with flagellin (Supplemental Fig. 1), indicating that the inhibition was not due to blockade of flagellin binding to cells by Vi. Vi showed a dose-dependent binding to human monocytes and interacted with these cells through membrane prohibitin complex (Fig. 2A, 2B).

Identification of serum proteins interacting with Vi

Vi was incubated for 48 h at 37°C with different concentrations of serum in the presence of anti-Vi Abs. The precipitates were pelleted down at 12,000 × g for 20 min at 4°C, washed gently with serum-free RPMI 1640, and run in a 12.5% SDS polyacrylamide gel, followed by silver staining. The proteins were transferred to a polyvinylidene difluoride membrane, and molecules precipitated specifically with Vi were subjected to N-terminal protein sequencing.

Surface plasmon resonance

The interaction of Vi with hemoglobin was analyzed by surface plasmon resonance (SPR) using the BIAcore 2000 (GE Healthcare, Uppsala, Sweden) instrument. Purified human hemoglobin (1 μM solution in 10 mM sodium acetate buffer, pH 5.5) was immobilized on a CM-5 sensor chip using a standard amine coupling method. This coupling resulted in 2000 response units of immobilized protein on the flow cell. Binding of Vi to immobilized hemoglobin was continuously monitored in HBS running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20, pH 7.4). To evaluate binding, Vi was diluted in HBS buffer and analyzed at various concentrations at a flow rate of 30 μl/min in a BIAcore2000. An activated and blocked flow cell without immobilized ligand was used to evaluate non-specific binding. Results were calculated using BIA evaluation version 4.1 software (BIAevaluation). The readings obtained with nonprotein coupled flow cell were subtracted from those obtained with hemoglobin-immobilized flow cell.

Gel filtration chromatography

Vi was incubated with hemoglobin for 1 h at room temperature before subjecting it to gel filtration chromatography using BioSep-SEC-S 2000 column (Phenomenex, Torrence, CA) connected to a Shimadzu HPLC system (Shimadzu, Tokyo, Japan), with PBS as the mobile phase (flow rate, 0.25 ml/min). Alternatively, Vi alone was subjected to gel filtration chromatography. Fractions were checked for their proinflammatory activity on THP-1 cells. These fractions were also run in a 10% native polyacrylamide gel and transferred to an NC membrane. The presence of Vi in different fractions was visualized by bloting the membrane with Abs to Vi.

Transfection with TLR

HEK293-T cells were transfected with various TLR-2 constructs (human TLR-2, hTLR-2/TLR-1, hTLR-2/TLR-6; Invivogen, Toulouse, France) using lipofectamine 2000 (Invitrogen, Carlsbad, CA) as the transfection reagent. Stably transfected cell lines were obtained after selection with blasticidin-S-hydrochloride (10 μg/ml; Sigma-Aldrich).

Results

Vi inhibits TLR-induced cytokine responses from monocytes through interaction with the membrane-associated prohibitin complex

To analyze possible modulatory effects of Vi on inflammatory responses from monocytes, we chose the TLR-5 ligand flagellin as a model stimulus because of its major role in the induction of inflammatory responses with pathogenic Salmonella. Human monocytic cell lines (THP-1 and U937), human PBMCs, and mouse BMDCs showed reduced cytokine secretion when stimulated with flagellin in the presence of Vi (Fig. 1A–C). Treatment with Vi did not result in any detectable cell death in these cells. U937 cells incubated with Vi and subsequently stimulated with flagellin showed reduced activation of p38-MAP kinase and reduced degradation of IkB (Fig. 1D); the former has been previously shown to be critical for TLR-5–induced IL-8 secretion from human monocytes (24). The suppression of cytokine secretion was seen even when the polysaccharide was added to cells after stimulation with flagellin (Supplemental Fig. 1), indicating that the inhibition was not due to blockade of flagellin binding to cells by Vi.

Vi showed a dose-dependent binding to human monocytes and interacted with these cells through membrane prohibitin complex (Fig. 2A, 2B). Both prohibitin and BAP-37 could be immunoprecipitated with Vi in these cells when analyzed with prohibitin and BAP-37–specific Abs (Fig. 2B). The possibility that Vi might be pulling down mitochondrial prohibitin was ruled out because Vi was not internalized in these cells (up to 60 min of incubation at 37°C) plus immunoprecipitations were always performed with lysates prepared from intact viable cells incubated with Vi at 4°C.

The interaction with prohibitin complex of proteins in the membrane was essential for the anti-inflammatory capability of Vi, because knockdown of prohibitin expression using a mixture of two siRNAs targeted against exons 1 and 3 resulted in reduced binding of Vi to U937 (Fig. 2C, 2D), and consequently the inhibition in flagellin-induced IL-8 secretion mediated by Vi was also reduced (Fig. 2E). Knockdown of prohibitin expression also led to a reduction in the expression of BAP-37 protein without affecting its RNA levels (Fig. 2C, 2F), which occurs because prohibitin and BAP-37 are always present as a heterodimer and BAP-37 monomers undergo degradation in the absence of prohibitin (25). However, knockdown of prohibitin did not change constitutive expression of GAPDH, showing that siRNAs against prohibitin were target-specific (Fig. 3).
degradation of I-phosphorylation of MAPKs (p38 and ERK) and brought about phosphorylation of THP-1 with Vi in the presence of serum activated complement. Polymyxin B abolished LPS-induced responses but did not affect Vi-induced responses (Supplemental Fig. 5). To determine the nature of the molecule in serum that was responsible for transforming Vi into a proinflammatory molecule, because digestion with proteinase K abrogated the ability of serum to promote inflammatory responses with Vi (Supplemental Fig. 5). To identify the nature of the molecule in serum that was responsible for producing changes in Vi, an immunoprecipitation was performed by incubating this polysaccharide with FCS in the presence of anti-Vi Abs. Amino acid sequence analysis of precipitated components showed that Vi specifically interacted with hemoglobin α, apolipoprotein A-I, and fetuin (Fig. 4A; other molecules in the precipitate were also seen with anti-Vi Ab in the absence of serum and Vi), suggesting that one or more of these proteins might be involved in generating inflammatory responses with this polysaccharide and in preventing its anti-inflammatory activity. The ability to generate inflammatory responses was tested by stimulating THP-1 with Vi in the presence of hemoglobin, apolipoprotein A-I, fetuin, and BSA. The results showed that only hemoglobin could promote Vi-mediated cytokine secretion (Fig. 4B). Of the two chains of hemoglobin, β-chain was significantly more efficient than α-chain at potentiating Vi-induced IL-8 secretion from THP-1 (Fig. 4B). The interaction between Vi and hemoglobin was further established by SPR, in which Vi showed a dose-dependent binding to hemoglobin immobilized on a sensor chip (Fig. 4B). The binding with hemoglobin produced a change in the mobility of Vi in a nondenaturing gel. Being an extremely high-m.w. polymer, Vi showed poor migration into the gel. However, when it was incubated with hemoglobin or its β-chain, it migrated readily into the gel, as revealed by Western blotting with
from untreated cells; VI, IP from VI-treated cells.

quantitative reverse-transcription PCR using gene-specific primers. C, IP
amounts of endogenous mRNA for BAP-37 and GAPDH were analyzed by

experiments.

elated for each set. Data are representative of at least two independent

ELISA, and percent inhibition in flagellin-induced IL-8 by VI was calcu-

absence of serum for 24 h. IL-8 was determined in the supernatants by

serum-free medium and 1 h later stimulated with flagellin (1

hibitin knockdown U937 (shPhb) cells were incubated with VI (5

Accepted with the

A

B

C

D

E

F

FIGURE 2. Prohibitin is required for inhibition of TLR-5-induced IL-8 secretion from human monocytes by VI. A, Cells were incubated with
different concentrations of VI followed by anti-VI mAb. Subsequently,
cells were stained with PE-conjugated anti-mouse Ig Ab and analyzed by
flow cytometry. Shaded histogram shows staining with control cells incubated
with anti-VI mAb and PE-labeled anti-mouse Ig Ab. B, U937 cells were incubated with VI (10 µg/ml) in PBS at 4°C for 1 h. Cell lysates
were immunoprecipitated with anti-VI mAb preloaded on protein-G-
sepharose beads and immunoblotted with anti-BAP-37 Ab followed by
anti-prohibitin Ab. *Residual reactivity of BAP-37 Ab. C, U937 cells were
transfected with empty (vector control) or prohibitin shRNA (shPhb)
expression vectors. Cell lysates were blotted with Abs to prohibitin (Phb),
BAP-37, and GAPDH. D, Cells were incubated with different concen-
trations of VI followed by anti-VI mAb. Subsequently, cells were stained
with PE-conjugated anti-mouse Ig Ab for 1 h and analyzed by flow cy-
tometry. Numbers on the histograms represent mean fluorescence in-
tensities. Shaded histogram shows staining with control cells incubated
with anti-VI mAb and PE-labeled anti-mouse Ig Ab. E, Control and pro-
hibitin knockdown U937 (shPhb) cells were incubated with VI (5 µg/ml) in
serum-free medium and 1 h later stimulated with flagellin (1 µg/ml) in the
absence of serum for 24 h. IL-8 was determined in the supernatants by
ELISA, and percent inhibition in flagellin-induced IL-8 by VI was calcu-
lated for each set. Data are representative of at least two independent
experiments. F, Total RNA was prepared from U937 cells transfected with
an empty vector or prohibitin shRNA expression vector. The relative
amounts of endogenous mRNA for BAP-37 and GAPDH were analyzed by
quantitative reverse-transcription PCR using gene-specific primers. C, IP
from untreated cells; VI, IP from VI-treated cells.

anti-VI mAb (Fig. 4D), indicating a change in the physical state of
VI that likely resulted from disaggregation. This shift in the
migration of VI in the presence of hemoglobin was not readily ob-
erved with the α-chain of hemoglobin or with apolipoprotein A-I
(Fig. 4D). Moreover, the mobility shift was reduced when VI was
incubated with hemoglobin in the presence of anti-VI mAb (Sup-
plemental Fig. 6). Furthermore, gel filtration of VI-hemoglobin
mixture demonstrated the presence of a novel VI species that eluted
later than native VI, which normally comes out in the void volume.
This faster migrating VI, which retained reactivity with O-acetyl as
well as N-acetyl recognizing anti-VI mAbs (Fig. 4E; VI + Hb,
fractons 7 and 8), induced IL-8 secretion from THP-1 (Fig. 4F).
This species was not seen when VI alone was subjected to gel fil-
tration chromatography (Fig. 4E; VI). The binding of VI to hemo-
globin or its β-chain was also associated with a mobility shift in
hemoglobin (Fig. 4D). Hemoglobin also mimicked the ability of
serum to abrogate the inhibitory effect of VI on the inflammatory
response from flagellin-stimulated THP-1 (Fig. 4G).

Hemoglobin-modified proinflammatory VI engages TLR-2 to
activate cellular responses

The ability of VI to induce secretion of inflammatory cytokines in
the presence of serum was also observed with the LPS hyporesponsive
murine macrophage cell line GG2EE and ex vivo peritoneal macro-
phages isolated from LPS hyporesponsive C3H/HeJ mice; both
secreted IL-6 in response to VI (Fig. 5A). These data suggest that
TLR-4 is not involved in the induction of cytokines by VI. This
finding, combined with previous studies implicating TLR-2 in the
recognition of polysaccharides (28, 29), prompted us to analyze the
role of TLR-2 in inflammatory responses produced by VI. The se-
cretion of IL-8 from THP-1 stimulated with VI was specifically
blocked by anti-TLR-2 mAb (Fig. 5B); an isotype matched Ab did
not inhibit this response. These results suggested that VI might en-
gage TLR-2 on THP-1 cells to generate inflammatory responses.
The role of this TLR in the activation of cellular responses by VI was
confirmed by the ability of this polysaccharide to induce IL-8 se-
cretion in the presence of serum or hemoglobin from HEK293-
T cells transfected with TLR-2/TLR-1 (Fig. 5C, 5D). Non-
transfected HEK293-T did not produce any IL-8 with VI in the
presence or absence of serum. Furthermore, HEK293-T transfected
with TLR-2/TLR-6 responded poorly, compared with TLR-2/TLR-
1, and only at a higher concentration of VI in the presence of serum
(Fig. 5C). The induction of some amount of IL-8 from TLR-2/TLR-
1–transfected HEK293-T with VI in the absence of serum might be
due to higher sensitivity of these cells compared with THP-1, be-
cause these cells also responded better to the known TLR-2 agonist
Pam3CSK (data not shown). Serum-independent response with these
transfectants also suggested that the vaccine preparation might have
small amounts of pre-existing proinflammatory VI present in it.
Importantly, THP-1 and HEK293-T do not express any detectable
levels of hemoglobin receptor CD163 (30, 31). Therefore, hemo-
globin did not directly contribute to any intracellular signaling event
during VI-induced inflammatory responses; its action was primarily
directed at the modification of VI.

Acetyl groups are required for the induction of inflammatory
responses with VI

Acetyl groups have been shown previously to be important for
generating Ab response against VI (18). To study the role of these
functional groups in the induction of inflammatory responses, VI
was either partially deacetylated to remove acetyl from N-acetyl
groups (NDeVi) or fully deacetylated to remove acetyl from N-
as well as O-acetyl groups (DeVi). N- and O-deacetylation was
monitored by reactivity with anti-VI mAbs recognizing different
determinants on VI (11) (Supplemental Fig. 7). IL-8 secretion
from THP-1 was not observed with DeVi, suggesting that acetyl
groups were required for the induction of inflammatory responses
with this polysaccharide (Fig. 6A). Significantly, partially deace-
tylated VI (NDeVi), which retained most of the O-acetyl groups
(Supplemental Fig. 7), triggered IL-8 secretion from macrophages
(Fig. 6A), indicating that O-acetyl might be the critical functional
determinants involved in the induction of chemokine secretion by
VI. Consistent with these results, commercially available poly-
galacturonic acid, which is chemically similar to VI except for the
lack of acetyl groups, also did not trigger any IL-8 secretion from THP-1 cells (Fig. 6A). However, O-acetylation at C-2 was not sufficient to produce inflammatory responses, because O-acetylated polygalacturonic acid also did not activate any IL-8 secretion (Fig. 6A). Therefore, in addition to the presence of O-acetyl, a specific configuration of Vi that might be produced following its interaction with hemoglobin is likely required to impart proinflammatory character to this polysaccharide. Importantly, further analysis revealed that deacetylation of Vi was associated with its inability to interact with hemoglobin. DeVi did not cause a mobility shift in hemoglobin, nor did it bind to hemoglobin bound to a chip (Fig. 6B, 6C). Alternatively, NDeVi showed a dose-dependent binding to hemoglobin (Fig. 6B, 6C).

**Vi induces inflammatory responses in vivo**

To establish the proinflammatory character of Vi in vivo, cytokines were analyzed in mice (C57BL/6J) injected with Vi. IL-6 was detected in the sera, and IL-6 and IL-12p40 were detected in peritoneal exudates (Fig. 6D) of mice injected with Vi and NDeVi, but not in mice injected with DeVi. These results were consistent with the in vitro data showing that DeVi incapable of interacting with hemoglobin was defective at inducing IL-8 secretion from human monocytes (Fig. 6A).

**Discussion**

The immune system recognizes microbes through a number of membrane-associated and cytosolic sensors, including TLRs and NLRs. This sensing results in the induction of host responses that constitute key components of innate immunity and play a critical role in determining the magnitude and quality of T cell responses that provide long term immunity against pathogens. These responses are regulated through a variety of mechanisms, and many pathogens have devised ways to evade these responses, thereby promoting establishment of infection. The host-pathogen interactions involved in the induction of immune responses and their regulation during infection with *S. typhi* remain poorly understood because a suitable animal model for this pathogen is not available. Most of our current understanding of typhoid pathogenesis is based on studies performed in mice with *S. typhimurium*, which produces an infection that is analogous to human typhoid. However, considering that *S. typhimurium* does not produce typhoid in humans, the conclusions about human typhoid based on this mouse model need to be interpreted cautiously.

We reported previously that Vi capsular polysaccharide, which is expressed in *S. typhi* but not in *S. typhimurium*, can target the prohibitin family of molecules in IECs and bring down inflammatory responses during infection of these cells with *S. typhi*. However, it is worth noting that the in vivo studies presented in this report were performed in mice, and it is possible that the responses elicited in this model may differ from those observed in human subjects.
We also suggested that such a downregulation early in the gut could promote establishment of infection. Given that Vi is released in abundance during in vitro growth of S. typhi and it has also been reported in the sera of typhoid patients (32), and taking into account conserved expression of prohibitin in all cell types including immune cells, we reasoned that interaction of Vi with membrane prohibitin complex might also modulate immune responses from these cells during systemic dissemination of S. typhi. The results presented in this study demonstrate that Vi can inhibit inflammatory responses from monocytes and macrophages, which are believed to be the main cell type that harbor S. typhi during systemic infection. This inhibition was dependent on the interaction of Vi with a membrane complex containing prohibitin and was associated with downregulation of MAPK pathways of intracellular signaling. The inhibition mediated by Vi was lost in the presence of serum, a phenomenon that was previously observed with IECs as well (7). However, what was striking with monocytes was that the loss of inhibition in the presence of serum was associated with the induction of potent inflammatory responses with Vi. The molecule in serum responsible for bringing about this switch was found to be hemoglobin. Hemoglobin transformed Vi into a proinflammatory species that was a potent...
hemolysis in vitro (3, 35–37). Hemoglobin has been previously shown to play a decisive role in guarding the host against anti-immune activities of Vi. Hemoglobin is known to exist extracellularly at concentrations ranging from 115 to 155 µg/ml, and these levels could rise further after infection with Salmonella, which has been shown to cause hemolysis in vitro (3, 35–37). Hemoglobin has been previously shown to potentiate cellular responses with LPS and LTA (3, 4, 38, 39). However, a striking difference between those studies and the present one is that here hemoglobin unveiled proinflammatory capability of Vi and abrogated its anti-immune effects. This modulation by hemoglobin might have important consequences for the induction of inflammatory and innate immune responses during infection with S. typhi. It is possible that the anti-immune effect of Vi prevails during early stages of infection with S. typhi in the gut, whereas the proinflammatory effects become apparent in the course of systemic dissemination of the pathogen during which host hemoglobin would be more readily available. In the absence of an animal model, it is not possible to subject our present findings to in vivo testing. However, we believe that anti-inflammatory and proinflammatory effects of Vi with ex vivo cells derived from human peripheral blood in the absence and presence of serum, respectively, provide sufficient evidence for a potent modulatory ability of circulating hemoglobin in preventing anti-immune activities of this virulence polysaccharide. The ability of hemoglobin to transform Vi into an immune activator is also supported by the
inability of deacetylated derivative of Vi incapable of interacting with hemoglobin to produce inflammatory responses in vitro and in vivo. The proinflammatory character of Vi might also contribute to the switching of anti-Vi Abs to IgG during vaccination with this polysaccharide, because immunization of mice with Vi produced IgM and IgG Abs (R. Garg and A. Qadri, unpublished data); IgG Abs correlate with the protective efficacy of the Vi vaccine (40).

The immune inhibition mediated by circulating Vi through interaction with membrane prohibitin in monocytes would be in addition to the recently reported effects of bacterial surface-associated Vi on regulating the accessibility and/or release of proinflammatory effectors from Salmonella, hence diminishing inflammatory responses (41, 42). Our findings reveal a previously unappreciated role for circulating hemoglobin in converting a key anti-immune virulence factor of S. typhi into an immune activator.

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Disclosures

The authors have no financial conflicts of interest.

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