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Monoclonal Antibodies of a Diverse Isotype Induced by an O-Antigen Glycoconjugate Vaccine Mediate *In Vitro* and *In Vivo* Killing of African Invasive Nontyphoidal *Salmonella*

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Nontyphoidal *Salmonella* (NTS), particularly *Salmonella enterica* serovars Typhimurium and Enteritidis, is responsible for a major global burden of invasive disease with high associated case-fatality rates. We recently reported the development of a candidate O-antigen–CRM_{197} glycoconjugate vaccine against *S.* Typhimurium. Here, using a panel of mouse monoclonal antibodies generated by the vaccine, we examined the relative efficiency of different antibody isotypes specific for the O:4 antigen of *S.* Typhimurium to effect *in vitro* and *in vivo* killing of the invasive African *S.* Typhimurium strain D23580. All O:4-specific antibody isotypes could mediate cell-free killing and phagocytosis of *S.* Typhimurium by mouse blood cells. Opsonization of *Salmonella* with O:4-specific IgA, IgG1, IgG2a, and IgG2b, but not IgM, resulted in cell-dependent bacterial killing. At high concentrations, O:4-specific antibodies inhibited both cell-free complement-mediated and cell-dependent opsonophagocytic killing of *S.* Typhimurium *in vitro*. Using passive immunization in mice, the O:4-specific antibodies provided *in vivo* functional activity by decreasing the bacterial load in the blood and tissues, with IgG2a and IgG2b being the most effective isotypes. In conclusion, an O-antigen–CRM_{197} glycoconjugate vaccine can induce O-antigen-specific antibodies of different isotypes that exert *in vitro* and *in vivo* killing of *S.* Typhimurium.

Invasive nontyphoidal *Salmonella* (iNTS) disease predominately manifests as bacteremia in sub-Saharan Africa and is most commonly caused by *Salmonella enterica* serovars Typhimurium and Enteritidis (1–3). With case-fatality rates of 20 to 25% in children (1) and up to 47% in HIV-infected adults (4), iNTS disease represents a major global health burden. Many African NTS isolates are multidrug resistant (5, 6), making iNTS disease difficult to manage. There is currently no vaccine against NTS for use in humans.

The presence of specific antibodies (Abs) against the O antigen of lipopolysaccharides (LPS), in addition to the outer membrane proteins and flagellar protein, has been well documented in response to *Salmonella* infection (7, 8). O antigen forms the distal portion of LPS and is a component of the outer membrane of Gram-negative bacteria. NTS bacteria that do not express O antigen are avirulent (9, 10). Due to the surface location of the O antigen and the almost universal presence of this antigen among pathogenic *Salmonella* strains, there is much interest in the potential of O antigen as a vaccine candidate (11). Immunization with experimental *Salmonella* O-antigen conjugate vaccines can effect *in vivo* killing of *S.* Typhimurium following an intraperitoneal challenge (12).

To address the need for a vaccine against iNTS disease, we recently developed a candidate *S.* Typhimurium O-antigen–CRM_{197} glycoconjugate vaccine using O antigen from the invasive African *S.* Typhimurium isolate D23580 (13). Initial studies with our vaccine indicated good immunogenicity in mice. Vaccination induced high levels of antibodies with cell-free bactericidal activity against D23580 (13).

Bactericidal antibody has the potential to protect against iNTS disease in Africans (14), and serum bactericidal assays (SBAs) have been used to gauge *in vitro* activity of naturally induced antibodies against *Salmonella* in Africans and Asians (14, 15). The role of antibodies in phagocyte-mediated immunity against *Salmonella* has been demonstrated in various studies in mice (16, 17), and antibodies are regarded as having a key role in immunity to *Salmonella* infection (18, 19). Antibody opsonizes *Salmonella* for uptake into phagocytes, resulting in intracellular killing of the bacteria, and is essential for phagocytosis and intracellular killing in both mouse and human macrophages (20–22). Blood cells, in the presence of opsonizing antibody and complement, can mediate phagocytosis and kill African *Salmonella* isolates (21).

In this study, we investigate the potential efficacy and mechanisms of antibody-dependent killing induced by an *S.* Typhimu-

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TABLE 1 Selection criteria for monoclonal O-antigen antibodies

Antibody specificity	Binding to *Salmonella* isolate:					
	S. Typhimurium D23580, O:1,4,5,12	*S.* Typhimurium LT2, O:1,4,5,12	*S.* Enteritidis D24954, O:1,9,12	*S.* Enteritidis SL7488, O:1,4,12	*S.* Senftenberg 20050439, O:1,3,19	*S.* Agona 20071186, O:4,12
O:1	+	+	+	+	+	−
O:4	+	+	−	+	−	+
O:5	+	+	−	−	−	−
O:9	−	−	+	−	−	−
O:12	+	+	+	+	−	+
23 subcloned monoclonal Abs	+	+	−	+	−	+

rium O:4,5-CRM_{197} glycoconjugate vaccine against iNTS disease. We examine the *in vitro* cell-free and cell-dependent functions of the vaccine-induced antibodies and *in vivo* killing of *Salmonella* following passive transfer and *S.* Typhimurium challenge. At high concentrations, O-antigen-specific antibodies from African adults are associated with impaired serum killing of *S.* Typhimurium and can directly inhibit bacterial killing by serum from healthy adults (23, 24). We therefore also examine the ability of O-antigen-specific monoclonal antibodies to inhibit *Salmonella* killing.

MATERIALS AND METHODS

Ethical approval. Ethical approval for the use of serum samples in this study was granted by the Life and Health Sciences Ethical Review Committee of the University of Birmingham. Informed written consent was obtained from participants. The care and use of all mice were in accordance with United Kingdom Home Office regulations (United Kingdom Animals Scientific Procedures Act 1986). The mice were maintained under specific-pathogen-free conditions and matched by age and sex within experiments.

Bacterial isolates. *Salmonella* isolates were *S.* Typhimurium D23580, *S.* Typhimurium LT2, *S.* Typhimurium SL1344, *S.* Enteritidis D24954, *S.* Enteritidis SL7488, *S.* Senftenberg 20050439, and *S.* Agona 20071186. D23580, multilocus sequence type (MLST) ST313, is an invasive African isolate of NTS from Blantyre, Malawi, and is representative of most invasive NTS isolates in Malawi since 2002 (14, 25, 26). LT2 and SL1344 are commonly used *S.* Typhimurium laboratory isolates previously described by Hoiseth and Stocker (27). D23580 and LT2 express O:1, -4, -5, and -12. D23580 also expresses O acetylation on C-2/C-3 in the rhamnose residues (13, 28). D24954 is an invasive *S.* Enteritidis isolate from Blantyre, which expresses O:1, -9, and -12. SL7488 is a mutant Thirsk isolate expressing O:4 in place of O:9, in addition to O:1 and O:12 (29). 20050439 expresses O:1, -3, and -19, while 20071186 expresses O:4 and O:12 (30).

O-antigen-specific monoclonal antibodies. The generation of the O-antigen-specific monoclonal antibodies was performed by Absea Biotechnology Ltd. (Beijing, China). Briefly, mice were immunized subcutaneously with two 8-μg doses of *S.* Typhimurium D23580 O-antigen–CRM_{197} glycoconjugate (with Freund's complete adjuvant) on days 0 and 21 and another 8-μg dose of glycoconjugate (with Freund's incomplete adjuvant) on day 42. On day 56, mice were boosted intravenously (i.v.) with 8 μg of the glycoconjugate. The mice were killed 3 days later, and their spleens were processed for cell fusion to generate hybridomas. O-antigen-specific antibody-secreting hybridomas were screened by Absea using *S.* Typhimurium O-antigen enzyme-linked immunosorbent assay (ELISA), where O-antigen-specific antibody was detected by goat anti-mouse Ig alkaline phosphatase (AP)-conjugated antibodies (Sigma, Milan, Italy).

Positive hybridoma lines were subcloned to obtain single clonal populations of hybridomas with each clonal population secreting antibody of the same specificity. Following subcloning, O-antigen-specific hybridomas were first screened using O-antigen ELISA by Absea and then further screened at the Novartis Vaccines Institute for Global Health (NVGH) for antibody binding to *S.* Typhimurium using flow cytometry and bacterial agglutination. To ensure that monoclonal antibodies were specific for *S.* Typhimurium O antigen, we analyzed the antibody binding to six different *Salmonella* isolates (Table 1) by flow cytometry (see below). Flow cytometric data were verified by bacterial agglutination with the six bacterial isolates using the same selection criteria shown in Table 1. The antibody isotypes of the selected antibody-secreting clones were also characterized using a mouse isotyping kit (Sigma). Five O-antigen-specific antibody-secreting clones, of different isotypes, were chosen (IgA, IgM, IgG1, IgG2a, and IgG2b). We included a mouse nonspecific IgG2a isotype monoclonal antibody (eBiosciences, Hatfield, United Kingdom) as a control.

Anti-O-antigen antibody binding assay. For the screening of O-antigen-specific hybridomas, salmonellae in log growth phase were incubated with the hybridoma culture supernatants at a final bacterial concentration of 2×10^8/ml. Antibodies bound to the bacteria were detected with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG, IgA, and IgM antibody (Sigma) (14). Samples were read on a FACSCanto instrument, and data were analyzed using FlowJo. The mean geometric fluorescence in the FL1 channel was used to indicate anti-*Salmonella* antibody levels. The percentage of bacteria that are bound by anti-O-antigen antibody was determined from the FL1 channel (14).

K_d analysis of O-antigen-specific antibodies. The avidity of purified O-antigen-specific antibodies binding to *S.* Typhimurium O antigen was assessed using a Biacore 3000 system (GE Healthcare, Buckingham, United Kingdom) (23). O antigen was immobilized on a hydrophobic HPA sensor chip (GE Healthcare). The chip was washed with wash buffer (100 mM sodium chloride containing 10 mM HEPES buffer, pH 7.4), followed by two washes with 40 mM *n*-octyl glucoside (Sigma). The chip was coated with 1 mg/ml O antigen by passing O antigen across the chip surface for 30 min at a flow rate of 2 μl/min. The chip was then washed with 0.1 M hydrochloric acid and blocked with 0.1 mg/ml bovine serum albumin (BSA). After coating, the chip was washed using two injections of 0.1 M hydrochloric acid and left in 0.1 M hydrochloric acid for 2 min. Each O-antigen-specific antibody isotype was 2-fold serially diluted to generate a series of eight dilutions in wash buffer (100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 nM) and then passed across the chip surface for 10 min at a flow rate of 5 μl/min. The chip was left in the antibody solution for 10 min and then washed with a continuous flow of wash buffer for another 10 min at a rate of 5 μl/min. The chip surface was regenerated with 0.1 M hydrochloric acid. Antibody binding was measured by observing the change in the surface plasmon resonance (SPR) angle of the O antigen bound to the chip for each of antibody dilutions. K_d (dissociation constant) values were calculated by fitting the binding curves to the one-site binding (hyperbola) model (chosen as it gave the best fit) using the BiaEvaluation software (Biacore; GE Healthcare) and GraphPad Prism.

Human complement. Normal human serum contains *Salmonella*-specific antibodies (14, 31). To use the serum as an exogenous source of complement, we depleted serum obtained from healthy HIV-uninfected

adult volunteers, with no known history of *Salmonella* disease, of these antibodies by adsorption with *Salmonella*. Briefly, bacteria were washed with phosphate-buffered saline (PBS) and prechilled to 4°C before being added to freshly thawed serum at a final concentration of 5×10^9/ml. The suspension was incubated at 4°C for 30 min, after which the bacteria were removed by centrifugation. The serum underwent two more cycles of incubation with the same amount of fresh bacteria. It was then sterilized through a 0.22-μm filter and used immediately. As human complement was found to be the only complement source able to mediate bacterial killing in both the SBA and the blood cell killing assays, it was used as the complement source for all subsequent assays.

Serum bactericidal assay. Bacteria in log growth phase were incubated with O-antigen-specific antibodies and exogenous complement (either baby rabbit serum [BRS] [31] or *Salmonella*-specific antibody [Ab]-depleted human serum). Numbers of viable salmonellae were determined after 180 min. BRS was obtained from AbD Serotec (Kidlington, United Kingdom). For SBA examining the inhibition of serum bactericidal activity, bacteria were incubated with O-antigen-specific antibody and 50% normal human serum, which effects a 2-$\log_{10}$ kill at 180 min after serum exposure (14).

Blood cell killing assay. Killing of *Salmonella* by RPMI-washed peripheral blood cells was determined in a blood cell killing assay. The assay has been adapted from one previously described using human blood cells from healthy HIV-uninfected adults (21) for use with mouse antibodies and mouse blood cells isolated from naive C57BL/6 mice (Harlan, United Kingdom). Bacteria were opsonized with O-antigen-specific antibodies and exogenous complement (either BRS, naive mouse serum, or Ab-depleted human serum) at a 75% final volume for 20 min at room temperature at a final bacterial concentration of 10^6/ml. Under these conditions, cell-free killing of *S.* Typhimurium does not occur. Opsonized salmonellae were added to washed blood cells at a multiplicity of infection (MOI) of 1:1 (final bacterial concentration of 10^5/ml). After 180 min, the cells were lysed with 0.1% Triton X (Sigma) and numbers of viable bacteria were determined. For blood cell killing assays examining the inhibition of bacterial killing, bacteria were opsonized with O-antigen-specific antibodies (over a higher concentration range), Ab-depleted human serum, and 1:64-diluted *S.* Typhimurium-specific mouse immune serum, which gives a 1-$\log_{10}$ kill in a 180-min blood cell killing assay.

Phagocytosis assay. FITC-labeled bacteria were first opsonized with O-antigen-specific antibodies and Ab-depleted human serum for 20 min at room temperature at a final concentration of 10^9/ml (21) or 10^6/ml. The opsonized salmonellae were added to precooled blood cells (all blood cells; final blood cell concentration of 10^5/ml) at an MOI of 1,000:1 or 1:1 (final bacterial concentration of 10^8/ml or 10^5/ml, respectively) and incubated at 37°C for 10 min. Trypan blue (0.16%; Sigma) was added to each sample to quench the fluorescence of noninternalized bacteria. Cells were resuspended in fluorescence-activated cell sorting (FACS) lysing solution (BD Biosciences, Oxford, United Kingdom). Samples were read on a FACSCanto flow cytometer (BD Biosciences), and data were analyzed using FlowJo. The percentage of blood cells that had phagocytized salmonellae was determined from the FL1 channel.

Mouse infection. Six- to eight-week-old female *Nramp*$^{-/-}$ C57BL/6 mice (Harlan, United Kingdom) were used. Six groups of six mice were passively transferred with 0.1 mg antibodies i.v.: the five different O-antigen-specific antibody isotypes and a nonspecific isotype control antibody (IgG2a). At 24 h following passive transfer of antibodies, the six groups of mice were challenged with 10^7 D23580 cells i.v. (equivalent to $\sim 0.5 \times 10^7$/ml as a mouse has ~2 ml of blood). A high inoculum was necessary to examine blood clearance, as only a small blood volume could be taken at each time point in order to minimize reaction due to blood loss. *In vivo* killing of *Salmonella* was measured by blood clearance and bacterial loads in the liver and spleen. Mice were bled at time points 15, 30, 60, and 360 min postchallenge to assess blood clearance of bacteria. At 24 h postchallenge, the mice were killed. Spleens and livers were homogenized in water using a Colworth stomacher (West Sussex, United Kingdom). Viable bacterial counts in blood, spleens, and livers were determined by LB agar plating.

Statistical methods. Comparisons of data from different groups of mice were performed using the Mann-Whitney *U* test, with a *P* value of <0.05 considered to be significant.

TABLE 2 Avidity of O-antigen-specific monoclonal antibodies

Isotype	$K_d \pm$ SE (nM)
IgA	60.42 ± 5.29
IgM	14.73 ± 0.60
IgG1	54.82 ± 6.30
IgG2a	58.01 ± 6.76
IgG2b	25.65 ± 4.34

RESULTS

O-antigen-specific monoclonal antibodies. Mice were immunized with the *S.* Typhimurium D23580 O-antigen–CRM_{197} vaccine. Using ELISA, 103 out of 2,400 (4.3%) hybridomas were found to secrete antibodies against O antigen. These were further screened for binding to live *S.* Typhimurium by agglutination and flow cytometry, leading to the selection of 25 hybridomas for subcloning. Twenty-three of the 25 (92%) hybridomas were successfully cloned, and we screened their supernatants for antibody binding to different *Salmonella* strains. Using the criteria shown in Table 1, we determined the O-antigen specificity of the 23 monoclonal antibodies. All were found to be specific for the O-antigen factor 4 (O:4), which is expressed by *Salmonella* group B isolates, including *S.* Typhimurium. A final panel of five monoclonal antibodies was selected for use in this study, each having a different isotype—IgA, IgM, IgG1, IgG2a, and IgG2b. High-pressure liquid chromatography (HPLC)–size exclusion chromatography (SEC) and SDS-PAGE indicated that most of the IgA monoclonal antibody was in monomeric form with a minority present as dimers (data not shown).

Avidity of O-antigen-specific antibodies. We determined the avidity of the five O:4-specific monoclonal antibodies by SPR. The K_d values of the five antibody isotypes are shown in Table 2. All five monoclonal antibodies have high avidities in the 15 to 60 nM range, with the IgM antibody having the highest avidity.

Cell-free bactericidal potential of O-antigen-specific antibodies. We examined the cell-free bactericidal potential of the O:4-specific antibodies against *S.* Typhimurium D23580 and SL1344 by SBA, using either 75% BRS (31) or human complement as an exogenous complement source.

All five antibody isotypes showed cell-free bactericidal activity against D23580 (Fig. 1A and C) and SL1344 (Fig. 1B and D), although there was a lack of bactericidal activity at high antibody concentrations. The bactericidal antibodies included IgA and IgG1 isotypes, which have been reported not to be able to fix complement (32, 33). These data confirm that O:4-specific antibodies have bactericidal potential against *S.* Typhimurium, consistent with our previous data on antibody raised to *S.* Typhimurium D23580 O antigen (34) and extracted O-antigen-specific antibodies from African adults and healthy adults in the United States (35, 36).

Blood phagocyte killing mediated by O-antigen-specific antibodies. The ability of the O:4-specific monoclonal antibodies to mediate killing of D23580 and SL1344 by blood phagocytes was examined. Bacteria were opsonized for 20 min at room tempera-

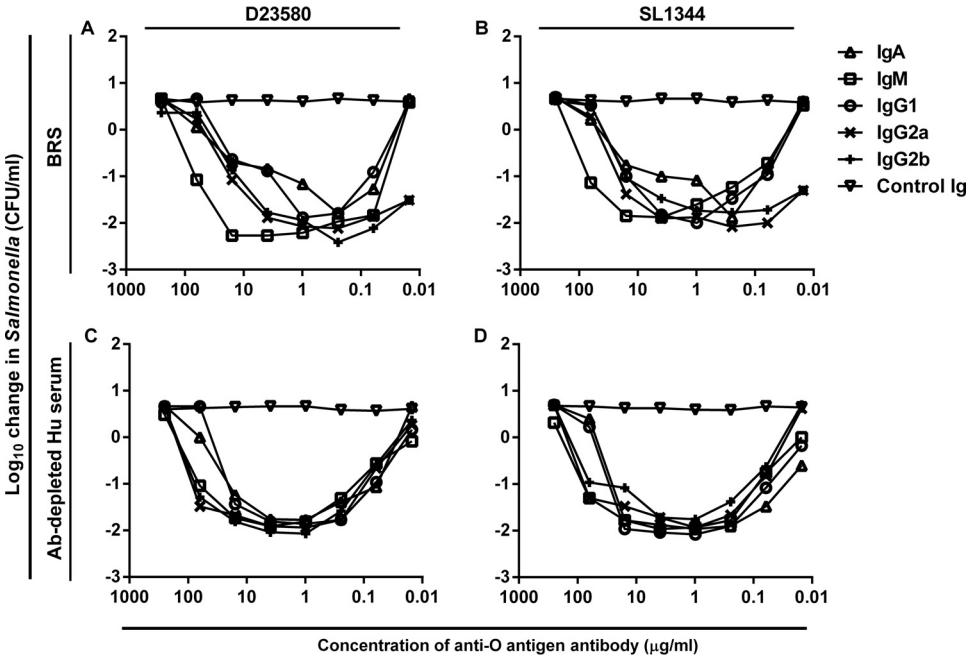

FIG 1 Cell-free killing of *S.* Typhimurium by O-antigen-specific antibodies. SBAs were performed with either BRS (A and B) or Ab-depleted human (Hu) serum (C and D) as a complement source. Target bacterial isolates were D23580 (A and C) and SL1344 (B and D). Mouse nonspecific IgG2a isotype was included as a control.

ture with the monoclonal antibodies and exogenous complement (conditions under which cell-free killing of *S.* Typhimurium does not occur) prior to incubation with peripheral mouse blood cells. We confirmed that the bacteria were opsonized by each of the antibody isotypes by examining antibody binding (Fig. 2A and B) using flow cytometry.

With 75% BRS as the complement source for opsonization, there was no blood cell killing of either D23580 or SL1344 (Fig. 3A and B), suggesting that BRS is not an appropriate complement source for *in vitro* bacterial killing by mouse antibodies and blood cells. When the complement source was 75% naive mouse serum (Fig. 3C and D), we observed an ~1-$\log_{10}$ kill of both isolates with IgA, IgG1, IgG2a, and IgG2b, despite a lack of killing at high antibody concentrations. IgM was unable to mediate bacterial killing by blood cells. With Ab-depleted human serum as complement source (Fig. 3E and F), again, O:4-specific IgA, IgG1, IgG2a, and IgG2b, but not IgM, could effect blood cell killing of *Salmonella*. These findings demonstrate that O-antigen-specific antibodies are able to mediate bacterial killing by blood phagocytes and reveal the isotype dependency of this.

Impact of high concentration of O-antigen-specific antibodies on cell-free bactericidal activity and phagocyte killing of *Salmonella*. Next, we examined whether high concentrations of the monoclonal antibodies could inhibit the killing of *Salmonella* by serum from healthy adults. All five monoclonal antibodies completely inhibited normal serum killing of D23580 (Fig. 4A) and SL1344 (Fig. 4B) at concentrations above 62.5 μg/ml. This inhibition occurred with all antibody isotypes.

We then examined whether the monoclonal antibodies could inhibit cell-dependent killing of *S.* Typhimurium by immune mouse serum. Immune mouse serum was diluted 1/64 and used to opsonize salmonellae, since it can effect blood cell killing of

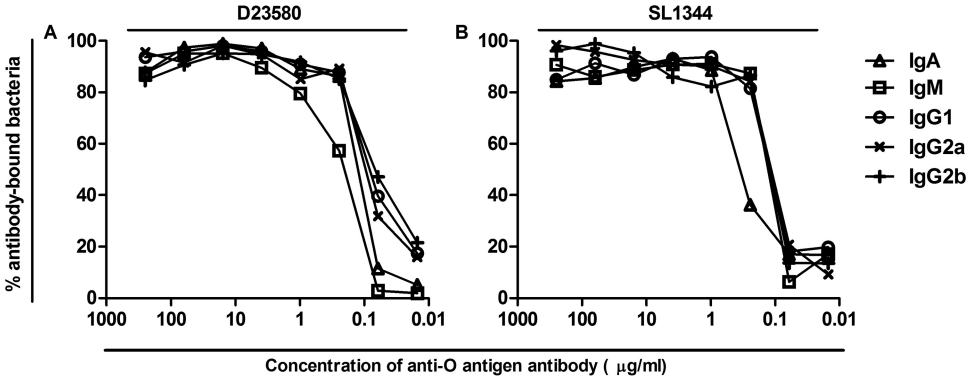

FIG 2 Binding of O-antigen-specific antibody on *S.* Typhimurium following opsonization. The percentage of O-antigen-specific antibody-bound *S.* Typhimurium D23580 (A) and SL1344 (B) following opsonization was determined by FITC-conjugated goat anti-mouse Ig antibody using flow cytometry.

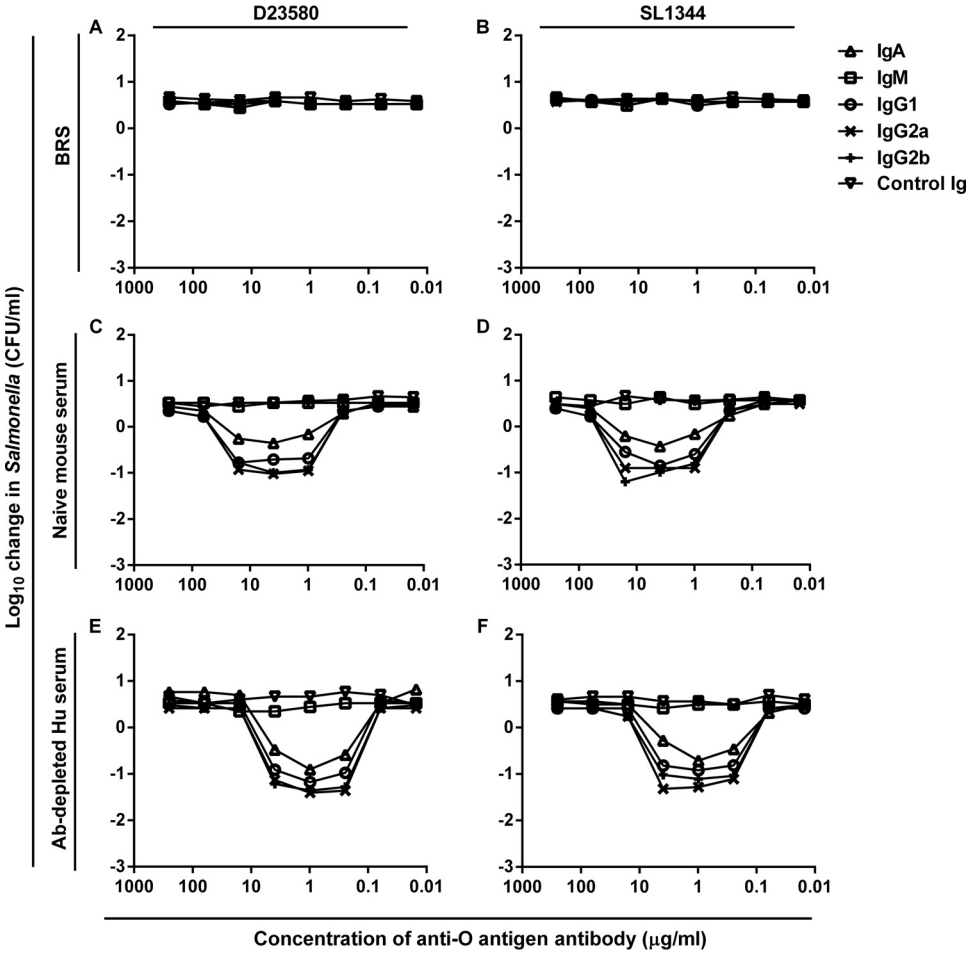

FIG 3 Blood cell killing of *S.* Typhimurium opsonized with O-antigen-specific antibodies and complement. Viable bacteria were opsonized with a mix of O-antigen-specific antibodies and BRS (A and B), naive mouse serum (C and D), or Ab-depleted human serum (E and F). Target bacterial isolates were D23580 (A, C, and E) and SL1344 (B, D, and F). Mouse nonspecific IgG2a isotype was included as a control.

D23580 and SL1344 by 1 $\log_{10}$ at this concentration (Fig. 5), together with human complement and a range of concentrations of purified O:4-specific antibodies. All O-antigen-specific antibodies inhibited cell-dependent killing of D23580 (Fig. 4C) and SL1344 (Fig. 4D) at concentrations of 8 μg/ml and above.

Phagocytosis of *Salmonella* opsonized with O-antigen-specific antibodies. We examined the ability of the O:4-specific monoclonal antibodies to mediate phagocytosis. When the bacteria were opsonized at a bacterial concentration of 10^9/ml and added to washed blood cells (all blood cells) at 10^8 bacteria/ml (MOI of 1,000:1; final blood cell concentration of 10^5/ml; final bacterial concentration of 10^8/ml), we observed efficient bacterial uptake with all isotypes of the monoclonal antibodies, with 80 to 90% of the blood cells phagocytizing bacteria opsonized with antibody at a concentration of 250 μg/ml and then gradually decreasing to levels comparable to those mediated by the control isotype at antibody concentrations of 0.24 μg/ml (Fig. 6A and B). Similar phagocytosis profiles were obtained with D23580 and SL1344.

We then repeated the phagocytosis assay, using bacteria opsonized at a concentration of 10^6/ml and added to washed blood cells (all blood cells) at 10^5 bacteria/ml (MOI of 1:1; final blood cell concentration of 10^5/ml; final bacterial concentration of 10^5/ml), as used in the blood cell killing assay. For both D23580 and SL1344, the maximal percentage of blood cells with phagocytized bacteria was ~30% and occurred when opsonized using all isotypes of O-antigen-specific antibodies at concentrations of 3.9 to 0.24 μg/ml (Fig. 6C and D). When high antibody concentrations of 250 to 16 μg/ml were used to opsonize the bacteria, in contrast to findings with 10^9/ml bacteria, the percentage of blood cells with phagocytized bacteria was greatly reduced using a bacterial concentration of 10^6/ml.

***In vivo* killing of *Salmonella* mediated by O-antigen-specific antibodies.** Using passive immunization, we investigated the ability of the O:4-specific monoclonal antibodies to kill *Salmonella* following systemic infection. All O:4-specific monoclonal antibodies mediated blood clearance efficiently, with the bacterial load over 2 $\log_{10}$ lower than with the control isotype antibody by the first time point at 15 min after bacterial challenge (Fig. 7). There was a further gradual decrease in the bacterial load between 15 and 60 min postchallenge. At 60 and 360 min postchallenge, the mice receiving the O:4-specific IgG2a and IgG2b antibodies had the lowest bacterial load. As mouse complement has limited bactericidal ability (37), the decrease in the bacterial load is likely to

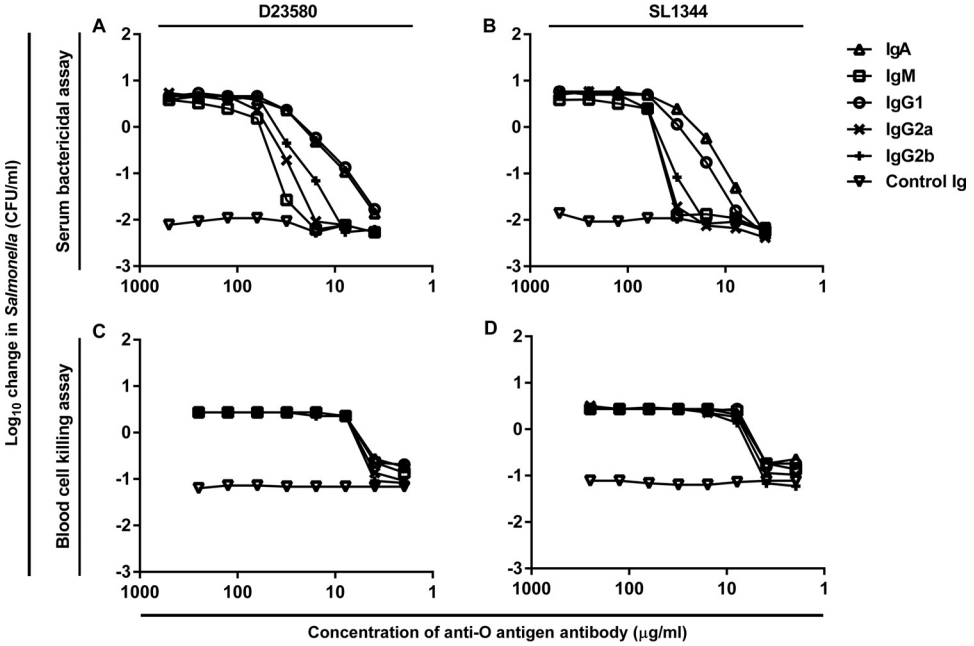

FIG 4 Inhibition of normal serum bactericidal activity and blood cell killing of *S.* Typhimurium by O-antigen-specific antibodies. Inhibition of serum killing of D23580 (A) and SL1344 (B) was examined in an inhibition SBA, where bacteria were incubated with different concentrations of O-antigen-specific antibodies and 50% normal human serum. Inhibition of blood cell killing of D23580 (C) and SL1344 (D) was examined in an assay of inhibition of blood cell killing, where bacteria were first opsonized with different concentrations of O-antigen-specific antibodies and mouse *S.* Typhimurium-immune serum at a 1/64 dilution.

result from bacterial uptake and bacterial killing by blood phagocytes and splenic macrophages.

At 24 h postinfection, mice receiving the O:4-specific IgA, IgG2a, and IgG2b ($P < 0.05$), but not IgM and IgG1, had lower bacterial loads in the liver (Fig. 7G) compared with the control isotype by approximately 0.5 $\log_{10}$. Passive transfer of all isotypes of the O:4-specific antibodies resulted in lower bacterial load in the spleen ($P < 0.05$) (Fig. 7H) compared with the control isotype by around 1.0 $\log_{10}$. IgG2a and IgG2b were the most efficient, with the lowest bacterial numbers compared to the other isotypes ($P < 0.05$).

DISCUSSION

O antigen is a principal target of the protective humoral responses against *Salmonella* (12, 29, 36, 38). Here, we have investigated the *in vitro* and *in vivo* killing and potential of the antibody response elicited by an *S.* Typhimurium O:4,5-CRM_{197} vaccine using monoclonal antibodies generated following immunization, with differing isotypes but all specific for the O:4 antigen of *S.* Typhimurium.

The first key finding is that antibodies specific to the O:4 antigen can effect cell-free serum killing of *Salmonella* and efficiently opsonize *Salmonella* for phagocytosis and bacterial killing by blood immune cells *in vitro*. These antibodies could also mediate *in vivo* killing following a virulent challenge of *S.* Typhimurium through blood clearance and reduced bacterial load in the spleen and liver. This is particularly important, since NTS bacteremia represents a major disease burden in Africa with high case-fatality rates (1, 4).

A previous study by Carlin et al. found that IgG1 and IgG3

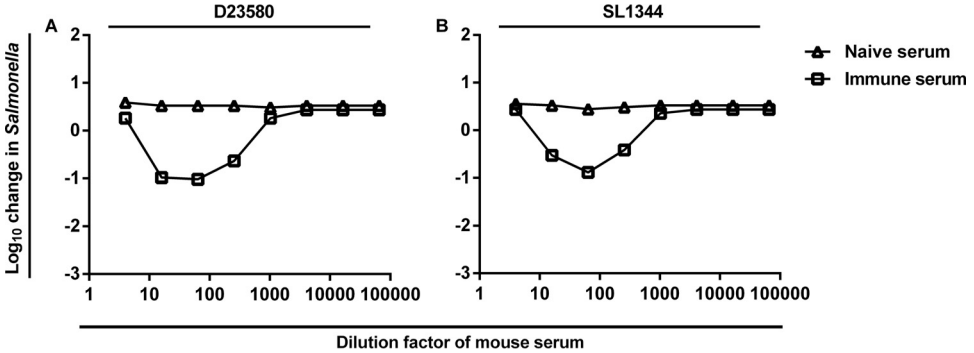

FIG 5 Blood cell killing of *Salmonella* mediated by naive and *S.* Typhimurium-immune mouse serum. Viable *S.* Typhimurium D23580 (A) and SL1344 (B) were opsonized with a mix consisting of Ab-depleted human serum and either naive mouse serum or *S.* Typhimurium-immune serum. Blood cells were incubated with the opsonized *Salmonella* at an MOI of 1:1 (final concentration of bacteria at 10^5/ml). After 180 min, the cells were lysed and numbers of viable bacteria were determined by serial dilution with plating on LB agar.

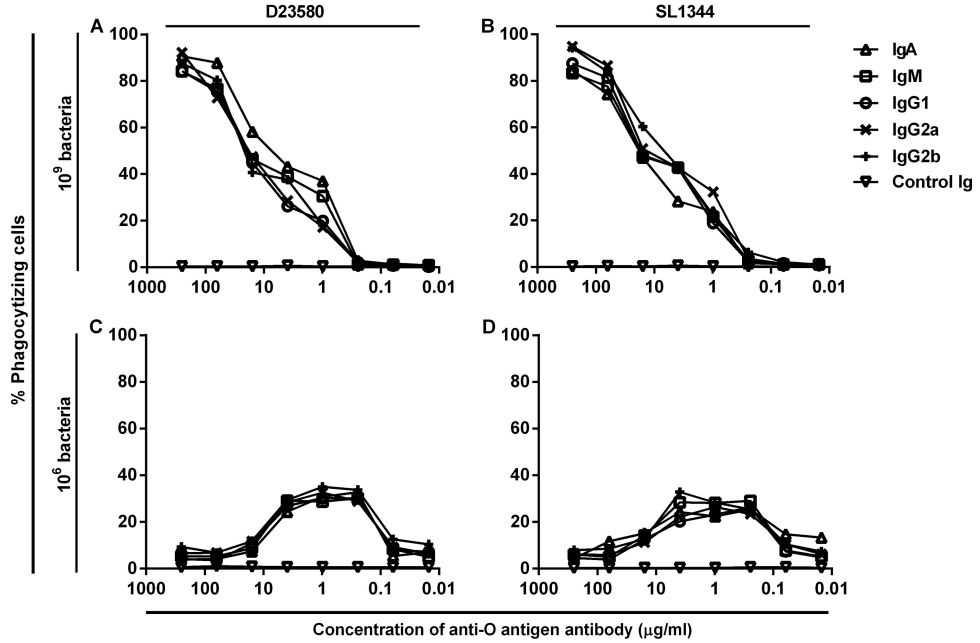

FIG 6 Phagocytosis of *S.* Typhimurium opsonized with O-antigen-specific antibodies and complement. FITC-labeled bacteria were opsonized with a mix of O-antigen-specific antibodies and Ab-depleted human serum at a final concentration of 10^9 bacteria/ml (A and B) or 10^6 bacteria/ml (C and D). Opsonized D23580 (A and C) or SL1344 (B and D) was added to precooled blood cells at an MOI of 1,000:1 or 1:1 (final concentration of bacteria at 10^8/ml or 10^5/ml, respectively). The percentage of blood cells that had phagocytized salmonellae was determined by flow cytometry.

monoclonal antibodies directed against the O:4 epitope of *S.* Typhimurium were potent at protecting mice against a 50% lethal dose (LD_{50}) intraperitoneal challenge with *S.* Typhimurium SH 2201 (38). IgG3 monoclonal antibodies to the O:12 epitope were much less effective. Since, in the current study, mice were challenged with the invasive African *S.* Typhimurium STS313 isolate D23580, our data help demonstrate the potential effectiveness of an *S.* Typhimurium O-antigen glycoconjugate vaccine for Africa. The lower reduction of bacterial numbers in the liver and spleen compared with that found in the blood may reflect the distribution of antibody in the body posttransfer and the half-life of the antibodies in these tissues.

The second key finding relates to the isotype dependency of O:4-specific antibodies able to mediate these *in vitro* and *in vivo* activities. Cell-free killing of *S.* Typhimurium was not isotype dependent. Since we have found that O-antigen-specific IgA in the blood of African adults does not have bactericidal activity (23), it is somewhat surprising that the mouse O-antigen-specific IgA can kill *S.* Typhimurium. It is plausible that IgA antibody from different species does not activate complement to the same degree. Previously, IgA has been reported to activate the alternative pathway (39, 40) and the mannose-binding lectin pathway of complement (41), suggesting a possible mechanism for the bactericidal activity that we observed in the study. The study by Carlin et al. (38) found that IgA monoclonal antibodies to *S.* Typhimurium O antigen did not fix complement and were not protective, but these were directed against the O:5 epitope, rather than the O:4 epitope.

Mouse anti-*Brucella* IgA and antihapten IgG1 have been reported to be incapable of fixing complement (32, 42). This could depend on the exact target of the antibody, or an O-antigen glycoconjugate vaccine may induce IgA and IgG1 with bactericidal potential, where natural exposure to *Salmonella* does not. For example, mouse transferrin-binding protein B-specific IgG1 has been reported to have bactericidal activity against *Neisseria meningitidis* (43). A study that examined the complement-fixing ability of mouse IgG antibodies targeting various antigens found six out of 17 mouse IgG1 antibodies able to activate complement efficiently (44).

In vitro cell-dependent killing of *S.* Typhimurium was isotype dependent, with IgM being unable to effect killing. However, all O:4-specific antibody isotypes were able to mediate phagocytosis with similar efficiencies. This apparently paradoxical finding could be due to alternative trafficking pathways in relation to phagolysosome fusion. In-depth studies to examine downstream signaling mediated by IgM Fc receptors could help clarify this. In addition to the polymeric Ig receptors that are mainly expressed by epithelial cells in the mucosal lumen (45), there are other IgM Fc receptors, Fcα/μR (46) and FcμR (47), which are also expressed by immune cells in the bloodstream. Fcα/μR has been reported to mediate phagocytosis of IgM-opsonized *Staphylococcus aureus* by B cells (46), while the recently identified FcμR has been suggested to serve as an uptake receptor for IgM-opsonized particles by B cells (47).

NTS carriage in asymptomatic individuals has been reported in Africa (48) and could constitute a reservoir for community-acquired NTS bacteremia in children (48). It is possible that NTS takes advantage of the inefficiency of IgM-mediated intracellular killing by blood immune cells to escape into the intracellular niche, where it has adapted to survive and can avoid antibody-dependent complement-mediated killing.

In vivo killing by the monoclonal antibodies following challenge with *Salmonella* was also isotype dependent. All O:4 antibody isotypes lowered bacterial loads *in vivo*. Despite the lack of killing in the *in vitro* blood cell killing assay, IgM lowered the

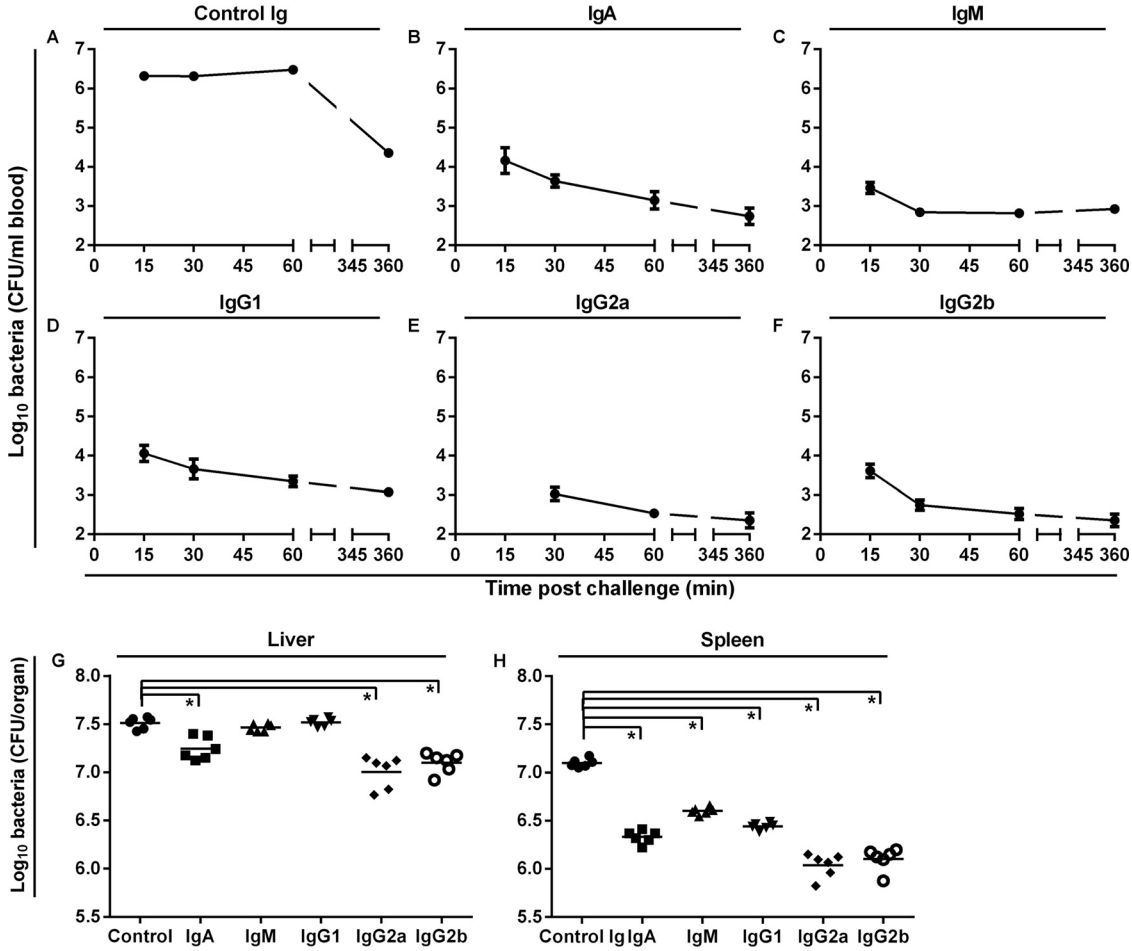

FIG 7 *In vivo* killing of *Salmonella* in mice following passive immunization with O-antigen-specific monoclonal antibodies and bacterial challenge. Bacterial loads in blood (A to F), liver (G), and spleen (H) were determined 24 h postchallenge in mice that were given 0.1 mg antibodies i.v., either control IgG2a antibodies (A) or IgA (B), IgM (C), IgG1 (D), IgG2a (E), or IgG2b (F) isotypes of the O-antigen-specific antibody 24 h before bacterial challenge. Each point in graphs A to F represents the mean $\log_{10}$ bacterial count ± standard deviation ($n = 6$), while each point in graphs G and H represents one mouse ($n = 6$), with the line representing mean $\log_{10}$ bacterial counts in each group of mice. *, P value of <0.05.

blood bacterial load in mice, possibly because IgM-mediated phagocytosis is as efficient as phagocytosis following opsonization with the other antibody isotypes. In the spleen, a lower bacterial load was observed with IgM than with the control isotype, which could be attributed to the different cell types involved, with IgM-mediated bacterial killing being more efficient in splenic macrophages than blood phagocytes. It is also plausible that there is more efficient bacterial killing mediated by IgM in the spleen, as it contains more B cells, than in peripheral blood, in which IgM receptors are found in greater abundance (46, 47).

IgM did not lower the bacterial load in the liver, and the reduction in bacterial load in the spleen was lowest following passive transfer with IgM, compared with the other monoclonal antibody isotypes. This suggests that IgM is less efficient in mediating *in vivo* protection than the other immunoglobulin isotypes. However, in the study by Carlin et al., IgM monoclonal antibodies to O:4,12 and O:12 were protective against the *S.* Typhimurium LD_{50} challenge (38). The difference in findings with IgM antibodies in the two studies may relate to differences in the challenge models used and differences in the specificities of the antibodies.

IgG2a and IgG2b in the current study were consistently the most efficient isotypes at lowering bacterial loads in the blood, liver, and spleen. This is likely because IgG2a and IgG2b efficiently bind to Fcγ receptors, particularly FcγRI, with high affinity (49). We have previously demonstrated that human IgG1 and IgG3 are more efficient in mediating intracellular *S.* Typhimurium killing *in vitro* (20). The production of IgG2a and IgG2b in mouse and IgG1 and IgG3 in human is upregulated by Th1 cytokines such as gamma interferon (IFN-γ) (50–52). Vaccine formulation and regimes that induce human Th1 responses resulting in a predominantly IgG1 and IgG3 antibody response could therefore be most effective in conferring protection against NTS.

The third key finding of the study is that there is an optimal range of O-antigen antibody concentration, beyond which *in vitro* killing activity against NTS is lost. Our phagocytosis data suggest that the *in vitro* opsonic function of antibody is dependent on the quantity of bacteria and the ratio of antibody to bacteria. The difference in the maximal percentage of blood cells with phagocytized bacteria in the two assays could be attributable to the difference in the MOIs. With a higher MOI, the maximal percentage of blood cells with phagocytized bacteria is likely to be higher. In addition, no O:4-specific antibody isotype was able to effect cell-

free bacteriolysis and cell-dependent bacterial killing at high antibody concentrations. When added to normal human serum and mouse immune serum at high concentrations, all O:4 antibody isotypes inhibited cell-free bacteriolysis and cell-dependent killing of *Salmonella*, respectively.

These data are consistent with our previous work on *S.* Typhimurium O-antigen-specific IgG antibodies from HIV-infected and HIV-uninfected African adults (23, 24) and recent studies on the IgG2 antibodies against the O antigen of *Pseudomonas aeruginosa* in patients with bronchiectasis (53). This loss of activity has been called a prozone effect and is an established phenomenon (54). Similar effects have been reported in the past, where passive transfer of high levels of specific antibodies has resulted in lack of protection against subsequent challenge with other pathogens, including pneumococcus (55) and *Cryptococcus neoformans* (54). Despite the *in vitro* inhibition observed in the current study, the O:4-specific antibodies were found to kill *Salmonella in vivo*, lowering the bacterial loads in blood, liver, and spleen, although only one dose of antibody was used. How these findings would impact the effectiveness of an O-antigen-based vaccine against iNTS for Africa is uncertain and will be answered only by appropriate clinical trials.

In conclusion, the O-antigen glycoconjugate candidate vaccine can induce antibodies that effect *in vivo* cell-free bacteriolysis and cell-dependent bacterial killing against *S.* Typhimurium, with efficient *in vivo* killing of *Salmonella* in mice, providing support for the idea that an O-antigen glycoconjugate vaccine is a promising vaccine approach against NTS.

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