Immunizations with Enterotoxigenic *Escherichia coli* Heat-Stable Toxin Conjugates Engender Toxin-Neutralizing Antibodies in Mice That Also Cross-React with Guanylin and Uroguanylin

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ABSTRACT Infection with enterotoxigenic *Escherichia coli* (ETEC) is a common cause of childhood diarrhea in low- and middle-income countries, as well as of diarrhea among travelers to these countries. In children, ETEC strains secreting the heat-stable toxin (ST) are the most pathogenic, and there are ongoing efforts to develop vaccines that target ST. One important challenge for ST vaccine development is to construct immunogens that do not elicit antibodies that cross-react with guanylin and uroguanylin, which are endogenous peptides involved in regulating the activity of the guanylate cyclase-C (GC-C) receptor. We immunized mice with both human ST (STh) and porcine ST (STp) chemically coupled to bovine serum albumin, and the resulting sera neutralized the toxic activities of both STh and STp. This suggests that a vaccine based on either ST variant can confer cross-protection. However, several anti-STh and anti-STp sera cross-reacted with the endogenous peptides, suggesting that the ST sequence must be altered to reduce the risk of unwanted cross-reactivity. Epitope mapping of four monoclonal anti-STh and six anti-STp antibodies, all of which neutralized both STh and STp, revealed that most epitopes appear to have at least one amino acid residue shared with guanylin or uroguanylin. Despite this, only one monoclonal antibody displayed demonstrable cross-reactivity to the endogenous peptides, suggesting that targeted mutations of a limited number of ST residues may be sufficient to obtain a safe ST-based vaccine.

KEYWORDS ETEC, diarrhea, enterotoxigenic *Escherichia coli*, enterotoxin, epitope mapping, guanylin, heat-stable toxin, immunological cross-reaction, uroguanylin, vaccine

Diarrheal diseases contribute considerably to the human disease burden in the developing world. Infections with enterotoxigenic *Escherichia coli* (ETEC) strains are estimated to cause approximately 25,000 deaths among children annually (1). Additionally, recurring ETEC infections are associated with malnutrition in children less than 5 years of age (2), and infections with ETEC are among the most common causes of traveler's diarrhea (3). For these reasons, the development of vaccines against ETEC is a priority (4), and several ETEC vaccine candidates are currently in different stages of development (5).

ETEC exerts its toxicity by secreting the heat-stable toxin (ST) and/or the heat-labile toxin (LT) into the small intestinal lumen (6). There are two ST variants found in ETEC strains infecting humans: the 19-amino-acid human ST (STh) and the 18-amino-acid

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porcine ST (STp), named after the species from which the ETEC strains were initially isolated (7, 8). STh and STp share 14 amino acids and have 3 disulfide bonds that define and stabilize their structure (7, 9). Importantly, ETEC strains that produce ST, with or without LT, have recently been identified as among the most important causes of moderate and severe diarrhea in children (10). Moreover, ETEC strains that produce STh are more closely associated with childhood diarrhea than those that produce STp (8, 11, 12). This implies that the STs in general, and STh specifically, are attractive targets for ETEC vaccine development, and ST-based vaccine antigens could be added to other ETEC vaccine candidate formulations to create vaccines with broad coverage (7).

The ST peptides act as superagonists of the guanylate cyclase C (GC-C) receptor, which is predominantly embedded in cell membranes of the cells lining the small intestine (13, 14). Following ST binding to the GC-C receptor, the receptor's intracellular cyclase domain is activated and catalyzes the conversion of GTP to cyclic GMP (cGMP), which leads to diarrhea through active export of electrolytes and subsequent release of water into the intestinal lumen through osmosis. While the activities of the GC-C receptor are normally regulated by guanylin and uroguanylin, these ligands bind the receptor with lower affinity than ST (14, 15).

The GC-C receptor with its regulatory ligands has recently also been implicated in several other key regulating functions, including appetite regulation (16), attention deficit hyperactivity disorder (17), increased blood pressure and altered electrolyte homeostasis (18), inflammatory bowel disease (19), ulcerative colitis disease (20), colonic hypersensitivity (21), and abdominal pain in patients with irritable bowel syndrome with constipation (22, 23). The fact that the STs are similar to guanylin and uroguanylin in both sequence and structure (9, 24–26) has led to concerns that anti-ST antibodies elicited by an ST-based vaccine component may cross-react with the endogenous ligands and thus possibly interfere with important physiological processes (27).

Three key challenges must be overcome to develop an ST-based vaccine: ST must be made immunogenic, it must be made nontoxic by mutation and still be able to elicit antibodies that neutralize native ST, and immunization with ST-based vaccines should not elicit antibodies that cross-react with the endogenous peptides (7). The nonimmunogenic property of ST has been thoroughly studied, and ST has previously been made immunogenic by coupling it to protein carriers by chemical conjugation or genetic fusion (7). Important progress has also been made on identifying ST mutants that have reduced or abolished toxicity but that still have the ability to elicit neutralizing antibodies (ST toxoids) when coupled to a carrier (28–30). The concern that immunization with ST antigens could elicit an unwanted immunological cross-reaction to the endogenous ligands was recently shown to be justified (26). In that study, both an anti-STh rabbit serum and an anti-STh monoclonal antibody (MAb), ST:G8, exhibited immunological cross-reactions toward uroguanylin (26). The dominant ST:G8 epitope residue is L9, which is shared with uroguanylin, and the epitope involves two more shared residues, E8 and N12 (28). Epitope mapping of the two non-cross-reacting anti-STp MAbs, C29 and C30, revealed epitopes centered around the ST-specific C-terminal tyrosine residue (28). These observations offer molecular insights that can be used to design ST-based vaccines with lower probabilities of eliciting cross-reacting antibodies. Interestingly, in contrast to the anti-STh antibodies, neither the anti-STp MAbs nor the anti-STp rabbit serum cross-reacted with the endogenous peptides (28). This suggests that STp could have a lower propensity for eliciting cross-reacting antibodies than STh, but due to the low number of samples that have been studied, further investigation is required.

To perform a systematic study of the immunological cross-reactive potential of STh and STp, we have immunized five mice each with native STh or native STp chemically conjugated to bovine serum albumin (BSA) and investigated the ability of the resulting sera to neutralize both STh and STp and to cross-react with the noncognate STs guanylin and uroguanylin. To acquire a better understanding of which residues are important for immunological cross-reactivity, we have also produced four new anti-STh

FIG 1 Anti-STh and anti-STp antibody titers and neutralizing abilities. (A) Titration of sera from the 10 mice was performed using ELISAs with coating with STh for anti-STh sera and STp for anti-STp sera. The titer was defined as the highest dilution with a signal-to-background ratio of ≥ 2.1 . (B) Neutralization of STh in the T84 cell assay by the anti-STh and anti-STp sera. The bars represent the mean normalized cGMP concentration for each dilution of sera relative to the peptide-only control (no serum). The error bars indicate the SD of the mean normalized cGMP concentration from five sera. (C) Neutralization of STp in the T84 cell assay by the anti-STh and anti-STp sera, performed and presented as in panel B.

MAbs and six new anti-STp MAbs, characterized their neutralizing ability and cross-reacting properties, and mapped epitope residues.

RESULTS AND DISCUSSION

Anti-STh and anti-STp mouse sera neutralize both STh and STp. In order to systematically investigate immunological cross-reactions of anti-STh and anti-STp antibodies, we immunized five mice each with STh or STp chemically conjugated to BSA. The anti-STh and anti-STp titers were estimated using enzyme-linked immunosorbent assays (ELISAs) with STh and STp peptide coating, respectively (Fig. 1A). The anti-STh sera had titers ranging from $10^{4.2}$ to $10^{5.7}$ (mean, $10^{5.3}$), and the anti-STp sera had titers ranging from $10^{5.4}$ to $10^{6.0}$ (mean, $10^{5.8}$). Preimmune sera were tested for all mice at a dilution of 1:1,000, but none of the observed ELISA results were above background levels, suggesting no or low levels of preexisting anti-STh and anti-STp antibodies. The sera were tested for the ability to neutralize both STh and STp in the T84 cell assay (Fig. 1B and C, respectively). At a 1:10 dilution, all sera completely neutralized 25 nM STh (Fig. 1B) and 25 nM STp (Fig. 1C). However, the differences in neutralizing ability of the sera at 1:100 and 1:1,000 dilutions suggest that the anti-STp sera contained higher titers of neutralizing antibodies than the anti-STh sera. The neutralizing titers varied between individual mice within each serum group, as reflected by the sizes of the error bars.

Anti-STh and anti-STp sera cross-react with uroguanylin and guanylin. Immunological cross-reactivity of the anti-STh and anti-STp sera was evaluated in competitive ELISAs in which wells were coated with native STh and STp, respectively, and in which we tested the ability of free STh, STp, uroguanylin, or guanylin (Fig. 2) to outcompete

FIG 2 Sequence alignment of STp, STh, uroguanylin, and guanylin. Cysteines are shown in light gray, and other residues shared by at least three of the peptides are in dark gray. The disulfide bonding pattern of STp and STh is shown above the alignment, and that of uroguanylin and guanylin is shown below. Residue numbers of STh are shown below the STh sequence, and percent sequence identity of each peptide to STh is shown to the right.

binding to the coating (Fig. 3). The noncognate ST peptides outcompeted binding to the coating of anti-STh sera 1 to 3 and anti-STp sera 2 to 5 comparably to the cognate ST peptides, suggesting strong immunological cross-reactivity. For a vaccine based on one ST variant, such cross-reactivity is advantageous since it may provide effective protection against both STh and STp. The inability of the noncognate ST peptides to completely outcompete binding to the coating of the remaining three sera (anti-STh sera 4 and 5 and anti-STp serum 1) demonstrates that the two ST peptides are sufficiently different to harbor distinct epitopes and that one may not consistently obtain full cross-protection by vaccinating with only one ST variant.

The human peptides uroguanylin and guanylin seemed to partially outcompete binding to the ST coating for all sera, with the apparent exception of anti-STh sera 4 and 5. In general, the sera displayed stronger immunological cross-reactivity to uroguanylin than to guanylin, which can be explained by the higher sequence similarity that uroguanylin has to the STs (26). These results corroborate the previously published observations that anti-STh antibodies can cross-react with uroguanylin (26) and demonstrate for the first time that both STh and STp immunogens can elicit antibodies that cross-react with both guanylin and uroguanylin.

To facilitate consistent comparisons of immunological cross-reactivity between sera, we used the 50 and 90% inhibitory concentrations (IC_{50} and IC_{90}) of the cognate peptides as common reference points (Fig. 3). The cross-reacting fraction of antibodies at these reference peptide concentrations was calculated by dividing the percent inhibition of each noncognate peptide with that of the cognate peptide (Fig. 4). The median cross-reacting fractions for the noncognate ST peptides were 0.77 (range, 0.51 to 1.24) at the IC_{50} (Fig. 4A) and 0.92 (range, 0.44 to 0.99) at the IC_{90} (Fig. 4B). The high median IC_{90} cross-reacting fraction suggests that most of the anti-ST antibodies in the sera cross-reacted with the noncognate peptides, and the lower IC_{50} cross-reactivity indicates that, on average, the anti-ST antibodies have poorer affinities toward the noncognate peptides. This is in line with the observation that the sera had a slightly poorer neutralizing ability toward the noncognate ST peptides (Fig. 1B and C).

The differences in cross-reacting fractions at the IC_{50} and IC_{90} for guanylin and uroguanylin are subtler: the median cross-reacting fractions for guanylin and uroguanylin at the IC_{50} ranged from 0.05 to 0.20, and at the IC_{90} they ranged from 0.10 to 0.23, suggesting low average immunological cross-reactivity. Cross-reacting fractions for guanylin in the anti-STh sera 3 and 4 could not be calculated due to having values close to zero, suggesting very low or no cross-reaction. Several individual sera, however, demonstrated strong immunological cross-reactivity, with the highest cross-reacting fractions observed between uroguanylin and anti-STh serum 3 and anti-STp serum 1, which had IC_{50} cross-reacting fractions of 0.65 and 0.71, respectively, and IC_{90} cross-reacting fractions of 0.55 and 0.53, respectively. The fact that the cross-reacting fractions are higher at the IC_{50} than at the IC_{90} suggests that these sera contain anti-ST antibodies that have high affinities toward uroguanylin.

Anti-STh serum #1 **Anti-STh serum #2** **Anti-STh serum #3** **Anti-STh serum #4** **Anti-STh serum #5** **Anti-STp serum #1** **Anti-STp serum #2** **Anti-STp serum #3** **Anti-STp serum #4** **Anti-STp serum #5**

FIG 3 Immunological cross-reactions of anti-STh and anti-STp sera were evaluated using competitive ELISAs. STh coating was used for anti-STh sera (anti-STh sera 1 to 5), and STp coating was used for anti-STp sera (anti-STp sera 1 to 5). All four GC-C receptor ligands were used in serial dilutions to compete for binding to the coating: STh, STp, guanylin, and uroguanylin. The vertical axes show percent inhibition of maximum binding to the ELISA coating, and the horizontal axes show peptide concentration on a logarithmic scale. Each data point represents the mean of three independent experiments, and the error bars depict SDs. The solid lines are regression curves from four-parameter logistic regression analyses. The dotted lines represent 50% (left) and 90% (right) inhibitory concentrations of the cognate ST peptide.

Anti-STh and anti-STp MAbs neutralize both STh and STp. Results from earlier studies suggest that anti-ST MAbs can provide useful information for the rational design of an ST vaccine (26, 28). Epitope mapping of one cross-reacting and one non-cross-reacting MAb revealed that differences in cross-reactivity could be explained by the presence or absence of shared amino acids in epitopes. With the aim to gain further insights into the molecular details of immunological cross-reactions, we gen-

FIG 4 Assessment of cross-reactive fractions at 50% inhibitory concentrations (IC_{50}) (A) and 90% inhibitory concentrations (IC_{90}) (B) of STh or STp. For each serum, four-parameter logistic regression was performed, and the IC_{50} and IC_{90} were calculated for the cognate peptides (i.e., STh for anti-STh sera and STp for anti-STp sera). These concentrations were used to calculate the inhibition for each peptide relative to that of the cognate peptides. The plots show the cross-reactivity at the chosen inhibitory concentration (vertical axis) for each peptide (horizontal axis) in each serum, and the lines depict median cross-reactions. Cross-reacting fractions for guanylin in the anti-STh sera 3 and 4 could not be calculated using regression analysis but had values close to zero (Fig. 3).

erated four new anti-STh and six new anti-STp MABs. The MAB-containing hybridoma cell line supernatants used in this study had comparable concentrations (mean, 48 [range, 38 to 57] ng/ml). All MABs were capable of neutralizing both STh and STp when tested in the T84 cell neutralization assay (Fig. 5). Some MABs completely neutralized both STp and STh (e.g., 7E52 and 21B1) at the concentration tested (1:10 dilution of supernatant), whereas others (e.g., 25G4 and 25B8) had lower neutralizing abilities. The fact that all MABs displayed neutralizing abilities makes them relevant as tools for developing ST vaccines.

Anti-STh and anti-STp MABs show little cross-reaction with uroguanylin and guanylin. Immunological cross-reactivity of the anti-STh and anti-STp MABs was measured by using competitive ELISAs in which all four GC-C receptor ligands were tested for the ability to compete for binding to immobilized STh or STp (Fig. 6). All MABs cross-reacted strongly with the noncognate ST peptide, as demonstrated by modest to no difference in affinities compared to the cognate peptide (with identical IC_{50} estimates or up to 4-fold differences), except for the two anti-STp MABs 25G4 and 25B8, which had >10-fold-lower affinities to STh than to STp (11-fold and 15-fold differences in IC_{50} estimates, respectively). Interestingly, these are the same two MABs

FIG 5 Anti-STh and anti-STp MABs neutralize both STh (A) and STp (B) in the T84 cell assay. The estimated cGMP production is given in percent relative to the negative control results (no MAB added). The bars represent the mean results from three separate experiments conducted with technical duplicates, and the error bars indicate the SDs. The MABs used in this assay were from hybridoma cell line supernatants, which had comparable MAB concentrations (mean, 48 [range, 38 to 57] ng/ml), and were used in 1:10 dilutions.

FIG 6 Immunological cross-reactions of anti-STh and anti-STp MAbs were assessed using competitive ELISAs. STh coating was used for anti-STh MAbs (7G11, 7E52, 25C5, and 23B7), and STp coating was used for anti-STp sera (29F6, 21F6, 23A7, 21B1, 25G4, and 25B8). All four GC-C receptor ligands were used in serial dilutions to compete for binding to the coating: STh, STp, guanylin, and uroguanylin. The vertical axes show percent inhibition of maximum binding to the ELISA coating, and the horizontal axes show peptide concentration on a logarithmic scale. Each data point represents the mean of three independent experiments, and the error bars depict SDs. The lines are regression curves from four-parameter logistic regression analyses.

that had the poorest neutralizing ability (Fig. 5). The anti-STh 7E52 MAb cross-reacted with uroguanylin, but with an IC_{50} of 12.3 μ M, which is more than 1,800-fold lower than that of STh (Fig. 6). The remaining MAbs had IC_{50} s for guanylin and uroguanylin at least 4 orders of magnitude lower than for the cognate peptides, suggesting no or very low affinities to these peptides.

Anti-STh and anti-STp MAb epitope mapping. To identify epitope residues for the different MAbs, we performed competitive ELISAs to test the ability of all possible variants of single-mutant STh peptides to compete for the binding of MAbs to immobilized native STh (28). We used STh mutants from our library of all possible 361 single-mutant STh peptides, excluding the 152 mutants that affect residues important

FIG 7 Epitope residue propensity score heat map of interactions between anti-STh and anti-STp MAbs and STh amino acid residues. The interactions were mapped by performing competitive ELISAs, in which single-mutant STh peptides were tested for the ability to compete for binding of MAbs to immobilized native STh peptides. The color intensity reflects to what extent the MAb binding was affected when a given amino acid was changed. A propensity score of 1 indicates that replacing the given amino acid with any other amino acid leads to at least a 50-fold reduction in ST binding, while a score of zero indicates that any change can be made to the given amino acid without affecting the binding. The STh amino acid residues that are shared with uroguanylin are marked with asterisks at the top. The two bottom rows indicate the minimum (MIN) and maximum (MAX) scores observed for each given amino acid residue across all MAbs.

for proper folding and/or effective production in *E. coli*, which were the N-terminal amino acid, the six cysteines in positions 6, 7, 10, 11, 15, and 18, and the proline in position 13. The interaction propensity scores for each MAb and amino acid combination depicted in Fig. 7 are based on the combined results from competition assays of all 19 amino acid variants. These scores reflect the extent to which the MAb binding is affected when a given STh amino acid is mutated. If all 19 mutant variants of a given STh residue bound a MAb with an affinity comparable to that of native STh, the propensity score was zero. The minimum propensity scores shown at the bottom of Fig. 7 demonstrate that for all the tested STh residues at least one MAb bound all the mutant variants of a given residue with near-native affinity, and hence these scores serve as negative controls. Conversely, the maximum propensity scores suggest that all tested STh residues, except for S2 and S3, are targeted by at least one of the MAbs. Note that since the epitope competitive ELISA screen was performed with STh ELISA coating and STh mutants, we do not know whether the six anti-STp MAbs also target the STp-specific T2 and F3 residues.

The 10 MAbs we generated displayed a wide range of interaction patterns, suggesting that they recognize a diverse repertoire of epitopes (Fig. 7). Interestingly, most epitopes have at least one high-scoring residue that is shared with uroguanylin, which could contribute to unwanted immunological cross-reactivity. However, four of the MAbs have the ST-specific Y19 residue as the highest-scoring residue, which may explain why these MAbs did not seem to cross-react with uroguanylin or guanylin. The anti-STh MAb 7E52 showed the clearest sign of cross-reactivity to uroguanylin (Fig. 6), and similar to the case with the previously characterized anti-STh MAb ST:G8, which also cross-reacted with uroguanylin (28), L9 is the central residue. The affinity of 7E52 to uroguanylin is, however, much lower than that of ST:G8, which may be explained by slight differences in epitope composition, where 7E52, in contrast to ST:G8, also interacts with the ST-specific residue Y19. A possible explanation for the lack of cross-reactivity with uroguanylin or guanylin for the remaining MAbs, except 25G4 and 25B8, is that their epitopes include at least one ST-specific residue. 25G4 and 25B8 appears to target shared residues, but further analyses are needed to determine whether they also target the STp-specific T2 and F3 N-terminal residues, which we have not included in these analyses.

Conclusions. There is evidence suggesting that exposure to certain virulence factors, either through natural infections or through vaccination, may induce autoimmune diseases. In some of these cases, autoimmunity is suspected to result from molecular mimicry in which antigens of the pathogen resemble host epitopes (31–33). Thus, avoiding potentially detrimental cross-reactive responses is a high priority when developing new vaccines (32–35).

In this study, we have expanded on previous studies which demonstrated that anti-STh antibodies can cross-react with uroguanylin (26), and we have now shown that both STh and STp can elicit antibodies that can cross-react with both uroguanylin and guanylin. However, there seem to be interindividual differences in the immune responses. All mouse sera, except two, demonstrated high cross-reactivity with the noncognate ST (Fig. 4B). The two exceptions, anti-STh sera 4 and 5, which only partially cross-reacted with STp, imply that an ST vaccine based on one variant might not offer equal protection toward both variants. We will, however, continue to focus on STh, due to the increasing evidence that ETEC strains that produce STh appear to be more pathogenic in young children than strains that produce STp (8, 11). The presence of antibodies that cross-reacted with uroguanylin or guanylin, observed to various degrees in the sera, suggests that mutation may be required to reduce the risk of eliciting antibodies with unwanted cross-reactivity.

The panel of MAbs investigated in this study demonstrated that it takes more than the involvement of individual shared residues to form cross-reacting epitopes. This is good news for the development of a safe ST vaccine, as it implies that it may not be necessary to change all shared residues to avoid unwanted immunological cross-reactivity. The main residue of the anti-STh 7E52 MAb epitope, which displayed the strongest cross-reactivity toward uroguanylin, was L9. Interestingly, L9 was also the main residue of the cross-reacting anti-STh ST:G8 MAb and seemed to be prominent also in the anti-STh rabbit serum that partially cross-reacted with uroguanylin (26, 28). Hence, L9 remains a prime target for mutation to avoid immunological cross-reactivity. Lastly, this study reiterates that optimizing the presentation of Y19-dominated epitopes may provide a path toward an ST vaccine that is safe from immunological cross-reaction (28).

To be broadly protective, an ETEC vaccine will probably need to target different stages of the ETEC infection process, including mucin degradation, adhesion, colonization, and toxin secretion. This implies that a multivalent ETEC vaccine will be required. There are several ongoing efforts to develop subunit vaccine components based on colonization factors and other conserved ETEC antigens, which could be combined with the highly immunogenic but nontoxic B subunit of LT (LTB) and an ST vaccine component (7).

MATERIALS AND METHODS

Preparation of ST peptides. The ST peptides were produced recombinantly as described previously (36). Briefly, STh and STp were expressed as genetic fusions to disulfide bond isomerase C (DsbC) and a 6×His tag. A tobacco etch virus (TEV) protease cleavage site located immediately before the STs allowed for the subsequent release of native ST without adding any amino acids from the cleavage site. The fusion proteins were expressed in *E. coli* BL21 Star (DE3) (Invitrogen, Waltham, MA) and, following cell lysis, purified with nickel-nitrilotriacetic acid (Ni-NTA) chromatography. Subsequently, the fusion proteins were incubated with 6×His-tagged TEV protease to cleave off the ST peptides from the fusion partner. A second Ni-NTA chromatography step was applied to remove uncleaved fusion proteins, the His-tagged fusion partner, and the TEV protease. The ST-containing flowthrough was loaded onto a preparative C₁₈ reverse phase column, and after elution with a methanol gradient, ST-containing fractions were pooled and concentrated using a vacuum centrifuge.

Preparation of ST conjugates. The purified STh and STp peptides were made immunogenic by conjugating to BSA by using glutaraldehyde (performed by GenScript, Piscataway, NJ). STh was also conjugated to LTB, as described previously (26). Briefly, 200 µg of STh was conjugated to 450 µg of LTB (Sigma-Aldrich, St. Louis, MO) by adding 600 µg of glutaraldehyde in 0.1 M phosphate buffer (0.1 M NaH₂PO₄, 0.1 M Na₂HPO₄ [pH 6.8]) in a total volume of 1 ml. After incubation for 120 min with gentle stirring in the dark at room temperature, the reaction was stopped by addition of 10 µl of 1 M L-lysine and further incubation for 30 min at room temperature. The completed reaction was dialyzed against phosphate-buffered saline (PBS) (3.25 mM Na₂HPO₄, 9.6 mM NaH₂PO₄, 146 mM NaCl [pH 7.4]) for 24 h at 4°C using a 10-kDa-molecular-weight-cutoff (MWCO) dialysis membrane (Spectrum Laboratories Inc., CA).

We followed the same procedure to produce STh- and STp-ovalbumin conjugates for use in the ST ELISAs (described below).

Regular and competitive ST ELISAs. All ST ELISAs were performed as described previously (26, 28). Briefly, Nunc Immobilizer amino plates (Thermo Fisher Scientific, Waltham, MA) were coated overnight at 4°C with 20 ng of STh-ovalbumin conjugates in 100 μ l of PBS for anti-STh antibodies or with 50 ng of STp-ovalbumin conjugates in 100 μ l of PBS for anti-STp antibodies. The wells were emptied and subsequently blocked by adding 180 μ l of 1% (wt/vol) ovalbumin (Sigma-Aldrich) in PBS-T (PBS, 0.05% Tween 20). All subsequent incubations were performed with gentle shaking for 60 min at room temperature, followed by three washes with PBS-T. For regular ST ELISAs, anti-ST serum or MAb was diluted to the required concentration in PBS-T, and 120 μ l was added to each well. For competitive ST ELISAs, peptide (STh, STp, guanylin, or uroguanylin) and antibody (anti-ST serum or MAb) were added to the STh-ovalbumin-coated wells in a total volume of 120 μ l. Following incubation and washes, 100 μ l of 1:4,000-diluted alkaline phosphatase-conjugated rabbit anti-mouse IgG secondary antibody (Abcam, Cambridge, UK) or 100 μ l of 1:2,000-diluted alkaline phosphatase-conjugated rabbit anti-mouse IgG secondary antibody (Sigma-Aldrich) was added. After incubation and washes, 100 μ l of substrate (250 mM diethanolamine, 0.5 mM MgCl₂, 0.5 mg/ml of 4-nitrophenyl phosphate disodium salt [pH 9.8]) was added, and the absorbance at 405 nm was measured within 30 min using a Hidex Sense microplate reader (Hidex, Turku, Finland). Background signal was subtracted from all measured absorbance values. The absorbance observed in wells containing competing peptides was converted to relative percent inhibition of maximum binding by dividing by the absorbance observed in the absence of a competing peptide.

Mouse immunizations and hybridoma cell line production. Mouse immunizations and hybridoma cell line production were performed by GenScript (Piscataway, NJ). In brief, two groups of five BALB/c mice were immunized subcutaneously with 50 μ g of STh-BSA or STp-BSA conjugate supplemented with Freund's complete adjuvant. After 14 and 28 days, the mice received booster doses of 25 μ g of conjugate supplemented with Freund's incomplete adjuvant. One week following the second booster, the mice were bled through the tail vein and the anti-ST antibody titers were determined as described below. In each group of 5 mice, the mouse with the highest anti-STh or STp IgG titers, as determined by ELISA, received a third booster injection intravenously with 25 μ g of ST-conjugate in PBS and was sacrificed after 4 days. Spleen cells from these mice showing signs of producing anti-ST antibodies, as determined by ELISA, were subsequently fused with myeloma cells to produce hybridomas. We obtained 2 anti-STh (25C5 and 23B7) and 6 anti-STp (29F6, 21F6, 23A7, 21B1, 25G4, and 25B8) cell lines. Using the same procedure, we additionally obtained 2 anti-STh cell lines (7G11 and 7E52) from mice immunized with STh-LTB conjugates.

Serum anti-STh and anti-STp IgG antibody titration. Serum from each immunized mouse was titrated for either anti-STh or anti-STp IgG antibodies in duplicates using regular ST ELISA as described above, except that we used 50 ng of STh or 50 ng of STp peptides for the coatings. Two-fold dilution series (from 1:1,000 to 1:2,048,000) of the sera were used in the assays, and the titers were defined as the last dilution in each series that had a signal-to-background ratio of ≥ 2.1 .

Monoclonal antibody production. Monoclonal antibodies were produced from hybridoma cell lines as described previously (37). Briefly, the 10 hybridoma cell lines were propagated at 37°C and 5% CO₂ in Nunc 24-well cell culture plates (Thermo Fisher Scientific) containing RPMI 1640 medium (Gibco Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 0.2% gentamicin (Lonza, Basel, Switzerland). When the cell density reached 30 to 50% confluency, the culture was transferred to 225-cm² cell culture flasks before continuing incubation until the culture was confluent. Each flask was then filled with medium and further incubated for 2 weeks or until most of the cells were dead. The culture was then centrifuged for 10 min at 145 $\times g$. The MAb concentration in each of the resulting supernatants was estimated by using a mouse IgG ELISA kit (Roche, Mannheim, Germany) on three dilutions of the supernatants according to the manufacturer's instructions.

Competitive ELISAs to determine immunological cross-reactions. Immunological cross-reactivity between anti-STh or anti-STp antibodies and STp or STh, respectively, as well as guanylin and uroguanylin was assessed using competitive ELISAs as described above. Three independent experiments, each conducted with technical triplicates, were performed for each sample. In these assays we diluted 10 μ M stocks of STh, STp, guanylin (Bachem, Bubendorf, Switzerland), or uroguanylin (Bachem) 3-fold 12 times, and 60 μ l of each peptide dilution was added to the ELISA plate wells, immediately followed by 60 μ l of antibody dilution. Antibody titrations were performed to identify optimal antibody dilutions for the competitive ELISAs. The mouse sera were diluted 1:1,000 and the MAb supernatants were diluted 1:30, except that MAbs 7G11 and 23A7 were diluted 1:90, 21F6 was diluted 1:3, 21B1 was diluted 1:810, 25G4 was diluted 1:5, and 25B8 was diluted 1:15. The competition was performed for 2 h at room temperature with gentle shaking.

T84 cell neutralization assay. The mouse sera and the 10 MAbs were analyzed for the ability to neutralize STh and STp as described previously (26). Briefly, T84 cells (CCL-248; ATCC, Manassas, VA) were seeded and grown to confluency in Nunc 24-well plates (Thermo Fisher Scientific) containing Gibco Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 0.2% gentamicin (Lonza, Basel, Switzerland). Cells were washed thrice with 500 μ l of DMEM/F-12 medium and preincubated with 200 μ l of DMEM/F-12 containing 1 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich) for 10 min at 37°C. We preincubated 10 ng of STh (24.4 nM final concentration) and STp (25.3 nM final concentration) overnight at 4°C with MAb supernatants or mouse sera diluted in 200 μ l of DMEM/F-12 medium. The MAb supernatants were diluted 1:3, while the mouse sera were diluted 1:10, 1:100, and 1:1,000. We added 100 μ l of sample to

each well and incubated the plate for 60 min at 37°C, followed by aspiration of the medium and cell lysis by incubation with 500 μ l of 0.1 M HCl at room temperature for 20 min. The lysates were centrifuged at 16,000 $\times g$ for 10 min and the cGMP levels were measured by using the direct cGMP ELISA kit (Enzo Life Sciences, Inc, Farmingdale, NY). We tested each peptide against each MAb in three independent experiments, each conducted with technical duplicates. The mouse sera were tested against each peptide once with technical duplicates.

Monoclonal antibody epitope mapping. Epitope mapping was performed by using competitive ELISAs as described above, except that filtered culture supernatants containing STh mutants from a library of all possible single-amino-acid mutant forms of STh, or native STh as a control, were used to compete for binding of MAbs to the STh-ovalbumin coating, as described previously (28). For the anti-STp MAbs 25G4 and 25B8, 25 ng and 12.5 ng of STh peptide, respectively, were used as coating. We excluded mutants of amino acid residues that are needed for proper folding and/or effective production in *E. coli*. Hence, we included mutants of STh residues S2, S3, N4, Y5, E8, L9, N12, A14, T16, G17, and Y19 in our analysis. Performing the analysis with technical triplicates for each given MAb-mutant combination, we mixed 60 μ l of diluted MAb supernatant (as described for the competitive ELISA above) with 60 μ l of STh mutant-containing filtered culture supernatant. For each STh mutant, we estimated the mutant's ability to inhibit binding to the STh coating relative to that of native STh. For each MAb and each STh amino acid, the relative inhibition values for all 19 mutants of that position were used to calculate an epitope propensity score, which was used as a measure of the strength of interaction between the MAb and the given residue. For each triplicate measurement, we calculated the mean fold inhibition for the given mutant-MAb combination and converted it to a mutant score of 0 (for <5.0-fold inhibition), 1 (for ≥ 5.0 - to <15.0-fold inhibition), 2 (for ≥ 15.0 - to <50.0-fold inhibition), or 3 (for ≥ 50.0 -fold inhibition). The epitope propensity score for the given MAb was then calculated by summing the 19 individual mutant scores and dividing by 57, which is the maximum possible score.

Data analyses and presentation. GraphPad Prism version 8 (GraphPad Software, La Jolla, CA) was used to plot all data. Competitive ELISAs were analyzed with four-parameter log-logistic regression in GraphPad Prism to fit data, plot inhibition curves, and estimate inhibitory concentrations (IC_{50}) using the following constraints: the bottom parameter was set to 0, and the top parameter was set to a maximum value of 100. To obtain an estimate of cross-reactivity between the cognate peptides (i.e., STh for anti-STh sera and STp for anti-STp sera), we calculated cross-reacting fractions. For these analyses, four-parameter log-logistic regression models were generated in R (drc R package [38]), using the same constraints. Based on the fitted models, we calculated the 50 and 90% inhibitory concentrations of each cognate peptide and used these concentrations to calculate the percent inhibition of binding for each of the noncognate peptides. The estimated values were adjusted by subtracting the bottom parameter estimate. Cross-reacting fractions were then calculated by dividing the adjusted percent inhibition value of a given peptide by the corresponding inhibition values for the cognate ST peptide. Data from two analyses (guanylin versus anti-STh serum 3 and guanylin versus anti-STh serum 4) could not be fitted to these models because of low responses to increasing peptide concentrations. For these two analyses, we consider the cross-reacting fractions to be zero.

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We declare no conflict of interest.

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