A Role for Stem Cell Factor and C-kit in the Murine Intestinal Tract Secretory Response to Cholera Toxin

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Summary

The role of stem cell factor (SCF) and its receptor (c-kit) in the intestinal secretory response to cholera toxin (CT) was investigated using a ligated intestinal loop model in mice having mutations in the dominant white spotting (W) locus and the steel (Sl) locus. W/W^v mice, which express an aberrant form of the c-kit protein, failed to give an intestinal secretory response after luminal CT challenge. In contrast, W/W^v mice and their control littermates had equivalent intestinal secretory responses to Escherichia coli heat-stable enterotoxin (STa). Sl/Sl^d mice, which express only a soluble truncated form of SCF, also gave a significantly reduced intestinal secretory response to CT when compared to the secretory response of their littermate controls. The unresponsiveness of W/W^v mice to CT was restricted to the intestinal tract since these mice had foot pad swelling responses to CT challenge that were equivalent to their littermate controls. Restoration of mast cells in W/W^v mice by bone marrow transplantation of control littermate bone marrow did not reverse the CT-unresponsiveness of the intestinal tract. Histological evaluation of the gastrointestinal tract from W/W^v mice showed a normal distribution of enterochromaffin cells (ECC). CT challenge of either ligated intestinal loops from C57Bl/6 mice or a mouse intestinal epithelial cell line (MODE-K) resulted in elevated levels of mRNA for SCF. MODE-K cells exposed to CT also had enhanced expression of c-kit. Finally, fluid obtained from CT-challenged ligated intestinal loops from C57Bl/6 mice contained significant levels of SCF. Taken together, the above results suggest that CT-induced intestinal secretory responses are dependent upon SCF–c-kit interactions. These interactions appear to be induced as a consequence of CT stimulation of the intestinal tract and may also play a role in the development or functionality of the enteric nervous system.

C holera is a diarrheal disease that is characterized by the hypersecretion of water and electrolytes from the patient's small intestine. The resulting diarrhea often causes hypovolemic shock and acidosis. Vibrio cholera is the causative agent of this disease and produces cholera toxin (CT), a potent enterotoxin, that mediates the fluid secretion and diarrhea associated with cholera (1, 2). Although CT has been investigated for many years, the exact molecular mechanism(s) involved in CT-induced fluid secretion is still not completely understood. CT has been shown to increase cellular adenylate cyclase which leads to increased intracellular concentrations of cAMP (1–4). The molecular mechanism of CT-induced fluid secretion is believed to involve this response as well as CT-induction of the synthesis and release of other cellular mediators including: prostaglandin E$_2$ (PGE$_2$) (4, 5), 5-hydroxytryptamine (5-HT) (6, 7), neurotensin (NT) (8), and platelet activating factor (PAF) (9). There is now strong evidence that fluid secretion induced by CT and other secretagogues also involves the enteric nervous system (10). CT-induced fluid secretion can be inhibited by a number of different nerve-blocking agents (10–14). Lundgren and colleagues (6, 7) have proposed that enterochromaffin cells (ECC) and other specialized epithelial cells in the epithelial lining of the small intestine release 5-HT and NT in response to CT which, in turn, activates

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[1] Abbreviations used in this paper: CHO, Chinese hamster ovary; CT, cholera toxin; ECC, enterochromaffin cells; ECL, enhanced chemiluminescence; G3PDH, glyceraldehyde-3 phosphate dehydrogenase; ICC, interstitial cells of Cajal; MODE-K, mouse intestinal epithelial cell line; NT, neurotension; PAF, platelet activating factor; PGE$_2$, prostaglandin E$_2$; SCF, stem cell factor; SI, steel locus; STa, Escherichia coli heat-stable enterotoxin; TBST, Tris-buffered saline plus Tween-20; W, white spotting locus.

secretory reflex(es) in the enteric nervous system. However, relatively little is currently known about the mechanism(s) by which the enteric nervous system functions with regard to fluid secretion or signaling via different cells or mediators. A potentially important component of the enteric nervous system is the innervated cellular network of the interstitial cells of Cajal. Interstitial cells of Cajal (ICC) are believed to generate electrical rhythmicity and mediate neural inputs in the gastrointestinal tract (15–17). Recently, ICC were shown to express the c-kit protein (17). The c-kit protein is a tyrosine kinase receptor of the PDGF/M-CSF receptor family and recognizes stem cell factor (SCF) as its ligand (18). Interestingly, mice with mutations in the dominant white spotting (W) locus, which have cellular defects in hematopoiesis, melanogenesis, and gametogenesis as a result of mutations in the kit gene (18), also lack ICC and intestinal pacemaker activity (19–21). These results indicate that c-kit–SCF interactions are necessary for the development of the ICC network. C-kit–SCF interactions have also been linked to the development and function of the central nervous system and may play a role in the formation of certain synaptic connections (18, 22, 23). Mast cell interactions with enteric nerves also appear to be necessary for fluid secretion associated with intestinal anaphylaxes (24–27). SCF is necessary for mast cell development and has been shown to activate mature mast cells (18, 28, 29). Thus, SCF–c-kit interactions could mediate potentially important regulatory functions for the enteric nervous system and play a role in the pathogenesis of enteric infections.

The possibility that c-kit–SCF interactions and/or the ICC network play a role in the intestinal tract secretory responses induced by CT or other enterotoxins has not been investigated. In this report we present evidence that the intestinal tract secretory response induced by CT is dependent upon c-kit–SCF interactions and that CT induces elevated levels of SCF production in the intestinal tract. Evidence is also presented that indicates that SCF–c-kit interactions are not necessary for intestinal secretory responses induced by *Escherichia coli* heat-stable enterotoxin (STa), and that STa- and CT-induced secretory responses may not be dependent upon the presence of the ICC network.

Materials and Methods

Reagents. CT and STa were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant rat SCF[1-164] (rrSCF) was expressed in and recovered from *E. coli* as previously described (30). Rabbit antibody to mouse SCF was generated by immunizing New Zealand white rabbits with Chinese hamster ovary (CHO) cell–derived recombinant mouse SCF[1-164]. The mouse SCF used as immunogen was expressed and purified essentially as described for CHO cell–derived recombinant rat and human SCFs (30). Immunoglobulin from pooled rabbit antisera was recovered using an Affigel Protein A MAPS II system (Bio-Rad Labs., Richmond, CA) according to the manufacturer's instructions.

Mouse Strains. All mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed under pathogen-free conditions. 8–14-wk-old female mice were used from the

following strains: C57Bl/6, WBB6F₁/J-W/Wᵛ, WCB6F₁/J-Sl/Slᵈ, and the respective littermate control mouse strains (WBB6F₁/J-++, WCB6F₁/J-++).

Ligated Intestinal Loop Assay. The ligated loop surgery was performed on mice as previously described (31, 32). Mice were fasted for 24 h before surgery. A midline abdominal incision was made to expose the small intestine, and a single segment (~5 cm) was ligated with 00 silk suture. Loops were injected with 100 μl of PBS containing CT or STa (1–2 μg per loop); control loops were injected with 100 μl of PBS. Incisions were closed with staples and mice were killed 6–7 h after surgery. At that time, loops were removed, measured, and fluid volume determined.

Mouse Foot Edema Assay. The procedure of Lexomboon et al. (33) was used to assess CT-induced footpad edema responses. Briefly, mice were anesthetized with ether and CT (1 μg in 10 μl of PBS) was injected into the hind paw with a Hamilton syringe fitted with a 27-gauge needle. The opposite paw was similarly injected with 10 μl of PBS. The thickness of the foot pad was measured in cm at various times with a digital caliper (Brown & Sharpe Mfg., North Kingstown, RI).

Bone Marrow Transplantation. WBB6F₁/J-WWᵛ and WBB6F₁/J-+/+ mice were given WBB6F₁/J-+/+ bone marrow cells intravenously at 2 × 10⁷ cells per mouse. After three months, mice were tested for CT-induced fluid secretion using the ligated loop assay and for the presence of mast cells by light microscopy as described below.

Histology. Small intestinal tissue from WBB6F₁/J-WWᵛ, WBB6F₁/J-+/+ and bone marrow transplanted W/Wᵛ mice were fixed in Carnoy's solution and stained with Toluidine blue for the detection of mast cells as previously described by Perdue et al. (27). Electron microscopy was used for assessing different intestinal tissues for the presence of ECC. In these experiments, small intestinal tissue was sliced into 2–3-mm cubes and fixed for 48 h in 5% glutaraldehyde diluted in PBS. The fixed tissue was reacted with a 1% aqueous solution of osmium tetroxide, before dehydrating in graded ethanol solutions and embedding in Polybed 812 (Polysciences, Warrington, PA). Thin sections of embedded tissue were stained with 2% uranyl acetate and lead citrate before examination in a Philips electron microscope for the general integrity of the epithelial tissue and for the presence of ECC containing polymorphous electron-dense granules.

MODE-K Cell Line. The MODE-K cell line is a mouse intestinal epithelial cell line that has been previously characterized (35). MODE-K cells were passaged routinely in DMEM medium containing 4.5 g/l glucose and supplemented with 10% heat-inactivated FCS, 4 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Confluent cell monolayers were detached three times a week, using a solution of 0.25% trypsin in 0.5 mM EDTA (Sigma Chemical Co., St. Louis, MO).

Northern Blot Analysis. The probe for murine SCF was a partial cDNA (34). A cDNA probe for the housekeeping gene glyceraldehyde-3 phosphate dehydrogenase (G3PDH) was obtained from Clontech Laboratories (Palo Alto, CA). The SCF-cDNA was nick translated in preparation of RNA hybridization. Northern blot analysis was performed as we have previously described (31, 32). Total mucosal RNA or MODE-K cell RNA was extracted using guanidine isothiocyanate and purified by cesium chloride gradient centrifugation. RNA samples were electrophoresed through a 1.4% agarose gel in the presence of formaldehyde then transferred to nylon membranes. Filters were UV cross-linked and baked at 80°C for 2 h and prehybridized for 18–24 h at 42°C in 6× SSPE, 5× Denhardt's solution, 0.5% SDS, 50% formamide, and 300 μg/ml denatured salmon sperm DNA. Hybridization

was performed at 42°C for 18–24 h in 6× SSPE, 5× Denhardt's solution, 0.1% SDS, 50% formamide, 100 μg/ml denatured salmon sperm DNA and 1 × 10⁶ CPM/ml [³²P]DNA. Initial washes were done at room temperature with 5× SSPE/0.5% SDS for 15 min, followed by 15 min under the same conditions. Filters then were washed twice in SSPE/0.5% SDS at 42°C for a total of 15 min. Autoradiography was performed with intensifying screens at −70°C by exposure of 1–3 d to XAR-5 film.

Flow Cytometry for Detecting c-kit. The expression of c-kit on MODE-K cells was assessed using flow cytometry. The following monoclonal antibodies were used for these experiments: (*a*) FITC-rat anti–mouse c-kit, (*b*) FITC–isotype control rat IgG, and (*c*) Fc receptor-blocking antibody, rat anti-CD32/16. All antibodies were purchased from PharMingen Inc. (San Diego, CA). Monolayers of MODE-K cells were established in T-25 flasks and after varying times of exposure to CT or medium were assessed for the expression of c-kit. One million cells were treated with Fc receptor blocking antibody and then with either anti–c-kit or isotype control Ig.

Dot Blot Analysis for SCF. A dot blotting assay using biotinylated rabbit antibody to SCF and a detection system using enhanced chemiluminescence (ECL) was developed for determining if SCF could be detected in fluids obtained from ligated intestinal loops challenged with CT. Fluids obtained from ligated loops were clarified by centrifugation and then total protein concentrations of each fluid determined. In these experiments, control loops were gavaged with a volume of PBS that was equivalent to the volume of fluid contained in the CT-challenged loops. Fluids were then diluted with PBS to 20 μg of protein per ml and 100 μl of this solution blotted onto nitrocellulose membranes using a Hybridot 96-well filtration manifold (GIBCO BRL, Gaithersburg, MD). For comparison, aliquots of PBS containing an equivalent concentration of FCS were spiked with varying concentrations of rSCF. Membranes were then blocked for 2 h with dry milk (10%) in Tris-buffered saline plus Tween-20 (TBST). Membranes were briefly washed twice in TBST and then four times for 15 min per wash in TBST. Blots were then exposed to purified and biotinylated rabbit IgG (2.5 μg/ml) specific for SCF. Blots were then washed as described above and exposed to streptavidin-HRP (Biosource International, Camarillo, CA). Blots were then washed and processed for ECL after standardized procedures of the manufacturer (Amersham Corp., Arlington Heights, IL).

Statistical Analysis. All numerical data were analyzed with a two-tailed Student's *t* test on independent samples. Data are presented as mean ± SE.

Results

Intestinal Secretory Responses of WWᵛ Mice. Ligated intestinal loops from WWᵛ mice and their control littermates (++) were challenged with CT and assessed for fluid secretion at 6–7 h after surgery. Control littermates responded to CT challenge with a typical secretory response (Fig. 1). In contrast, WWᵛ mice rarely gave a secretory response to CT challenge (Fig. 1). 13 of the 14 mice tested gave no fluid secretion in their ligated intestinal loops challenged with CT. The one mouse that did respond had only a marginal response (73 μl/cm) compared to their control littermates (168 ± 34 μl/cm). To investigate whether the abnormal secretory response of WWᵛ mice was unique to CT, ligated intestinal loops of WWᵛ mice and their control lit-
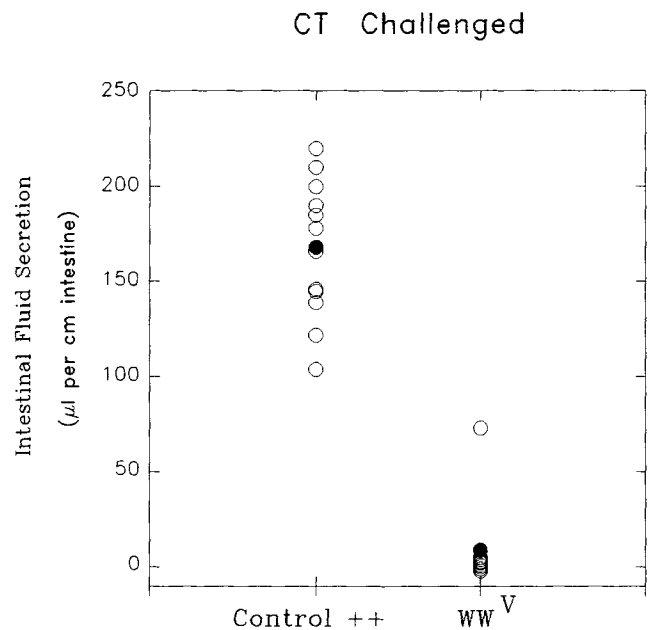

CT Challenged

Figure 1. Defective intestinal secretory response to CT by WWᵛ mice. Ligated intestinal loops from WWᵛ and control littermates (++) were challenged with CT and assessed for fluid secretion at 6–7 h after surgery as described in Materials and Methods. Fluid secretion from 14 control (++) mice and from 14 WWᵛ mice is presented. Mean fluid secretion is indicated by filled circles and was as follows: WWᵛ = 9 ± 9 and ++ = 168 ± 34. Fluid secretory responses between the mouse strains were statistically different at *P* <0.001.

termates were challenged with STa. STa is another enterotoxin that induces fluid secretion in ligated intestinal loops via guanylate cyclase activation and cyclic GMP formation (36–38). As seen in Fig. 2, WWᵛ mice and their control littermates responded to intestinal challenge with STa with equivalent levels of fluid secretion. These results indicate that WWᵛ mice have the ability to give intestinal secretory responses, but STa and CT mediate fluid secretion by mechanisms that are distinguishable using the WWᵛ mouse.

CT-induced Footpad Swelling Is Normal in WWᵛ Mice. CT has been shown to induce a secretory response in the footpad of rodents (33, 39). We investigated whether WWᵛ mice were unresponsive to CT at sites other than their intestinal tract. The hind paws of WWᵛ and their control littermates were injected with either CT or PBS. Footpad swelling then was assessed at 7 and 24 h. As seen in Fig. 3, WWᵛ and their control littermates had identical footpad swelling responses to CT.

Role of Mast Cells and ECC in CT Unresponsiveness by WWᵛ Mice. WWᵛ mice are mast cell deficient (18) but this deficiency can be corrected by transplantation of bone marrow obtained from littermate control mice (27, 40). To investigate whether the inability of WWᵛ mice to give intestinal secretory responses to CT was due to the lack of mast cells, we transplanted WWᵛ mice with bone marrow from their littermate control mice. Controls for this experiment were littermate control mice transplanted with an equivalent number (2 × 10⁷) of their own bone marrow cells. After
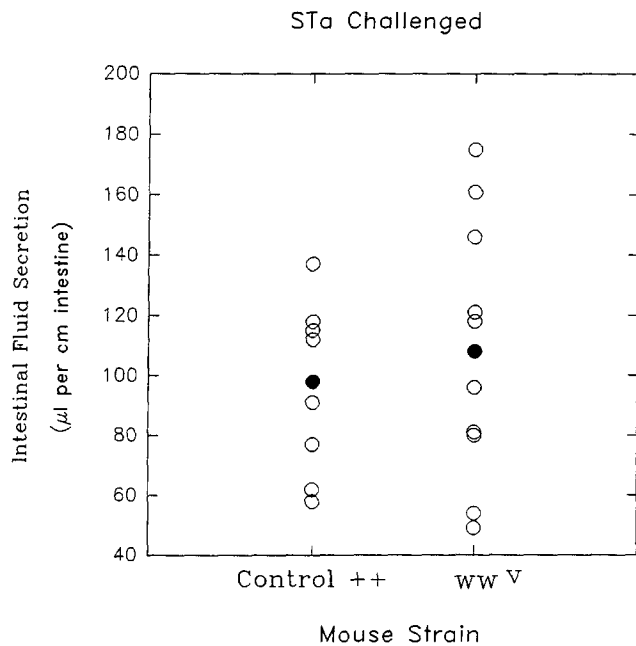
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STa Challenged

Figure 2. Intestinal secretory responses to STa are normal in WW^v mice. Ligated intestinal loops from WW^v and control littermates (++) were challenged with CT and assessed for fluid secretion at 6–7 h after surgery as described in the Materials and Methods. Mean fluid secretion is indicated by filled circles and was as follows: WW^v = 108 ± 12 and ++ = 98 ± 9. Fluid secretion from nine control (++) mice and eleven WW^v mice is presented. Fluid secretory responses between the mouse strains were not statistically different P = 0.63.

three months, transplanted mice were assessed for the presence of mast cells and for their secretory response to CT using the ligated intestinal loop assay. As seen in Fig. 4, tissue sections from the small intestine of WW^v mice contained no detectable mast cells (A and B). However, WW^v
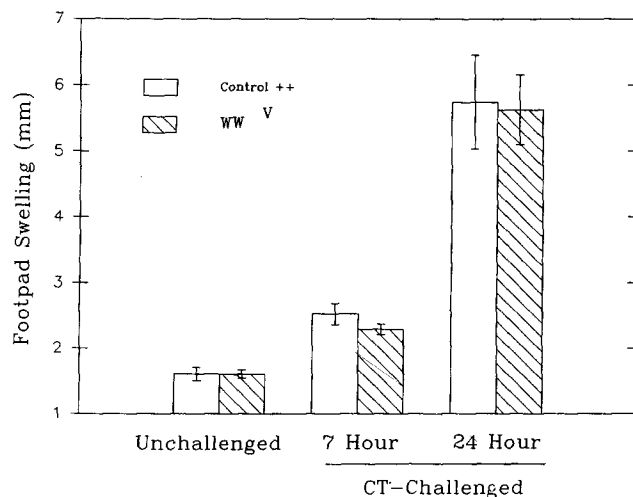

Figure 3. CT-induced footpad swelling is normal in WW^v mice. Footpad swelling responses were assessed in WW^v versus control littermates (++) at 7 and 24 h as described in Materials and Methods. Data presented are from five mice per mouse strain at each time point. CT-induced footpad swelling was not statistically different between the two mouse strains.

mice that had been transplanted with bone marrow from littermate controls contained a tissue distribution of mast cells that was identical to that of transplanted littermate control mice (Fig. 4, C–F). Ligated intestinal loops from transplanted WW^v mice and their transplanted control littermates were then challenged with CT and fluid secretion was measured. As seen in Fig. 5, mast cell reconstitution of WW^v mice by bone marrow transplantation had no effect on the CT unresponsiveness of the gastrointestinal tract. Ligated intestinal loops from bone marrow transplanted WW^v mice failed to give any fluid after CT challenge (Fig. 5). In contrast, bone marrow transplanted control mice gave a significant secretory response to CT (139 ± 8 µl per cm). These results indicated that the mast cell deficiency of WW^v mice was not the cause of the intestinal tract unresponsiveness to CT.

The ECC is another cell type believed to be important for CT-induced fluid secretion in the intestinal tract. Whether the development of ECC in the intestinal mucosa was also affected by the c-kit mutation present in WW^v mice was next considered. Small intestinal tissues from WW^v and their littermate controls were examined by electron microscopy for the presence of ECC. Examination of the small intestinal tissues from these mice revealed the presence of ECC that contained polymorphous electron-dense granules. Representative electron micrographs of ECC present in control mice (A) and WW^v mice (B) are presented in Fig. 6. These cells were present in thin sections of small intestinal tissue from WW^v and control mice in approximately equal numbers (2–3 ECC/grid) and had identical morphology that appeared to be completely normal.

Sl/Sl^d Mice Have Abnormal Intestinal Secretory Responses to CT. SCF is expressed as a transmembrane glycoprotein as well as in a soluble secreted form (41–44). Sl/Sl^d mice have mutations at the steel (Sl) locus that result in only the expression of a soluble truncated form of SCF (43–45). To investigate further the role of SCF and c-kit in CT-induced intestinal secretory responses, we investigated Sl/Sl^d mice for their ability to respond to CT. Ligated intestinal loops from Sl/Sl^d and their littermate controls were challenged with CT and assessed for fluid secretion. Results from four different experiments are presented in Fig. 7. Sl/Sl^d mice either failed to respond to CT or gave a secretory response that was significantly less than that of their littermate controls (Sl/Sl^d = 34 ± 51 versus controls = 158 ± 52). 10 of the 17 Sl/Sl^d mice challenged with CT failed to give a secretory response. A low but detectable secretory response was observed in 7 of the 17 Sl/Sl^d mice challenged with CT. However, the secretory response of these Sl/Sl^d mice was still significantly less than that given by their littermate controls (Sl/Sl^d 83 ± 48 versus controls 158 ± 52).

CT Induces Increased Levels of SCF mRNA in Ligated Intestinal Loops and in Intestinal Epithelial Cells. Results obtained from experiments using WW^v and Sl/Sl^d mice suggested that CT-induced fluid secretion may involve the production of SCF and its resulting interaction with c-kit–bearing cells. To explore this possibility, we assessed CT-challenged ligated intestinal loops for increased levels of SCF mRNA
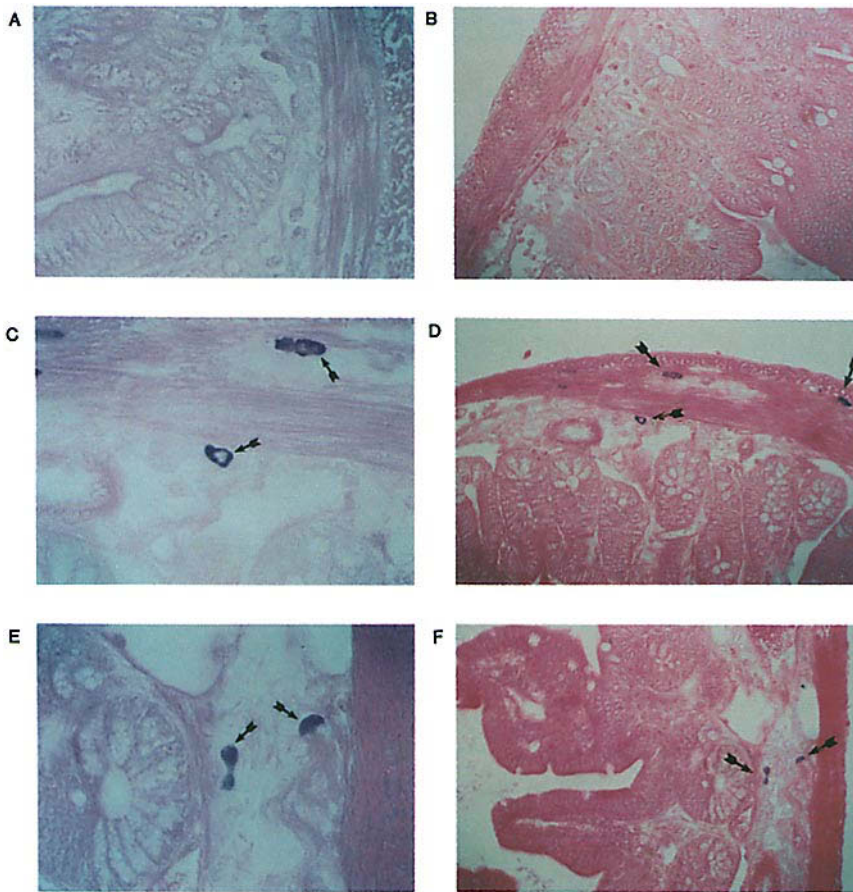
Figure 4. Mast cell repopulation of WW^v mice by bone marrow transplantation. Small intestine tissue sections were obtained from the following mice: WW^v (*A* and *B*), WW^v transplanted with ++ bone marrow (*C* and *D*), and ++ transplanted with ++ bone marrow (*E* and *F*). Tissue sections were processed as described in Materials and Methods. Arrows identify mast cells present in submucosa and lamina propria of small intestine. *A–F* are representative of the histology observed in each mouse. *A*, ×600; *B*, *D*, and *F*, ×200; *C* and *E*, ×1000.
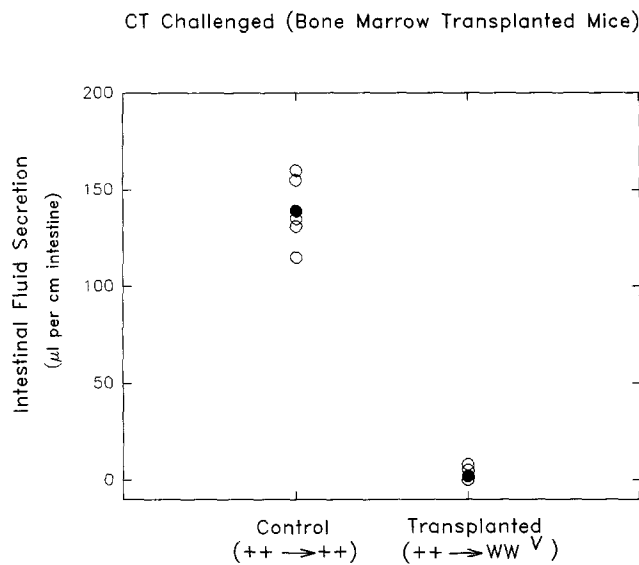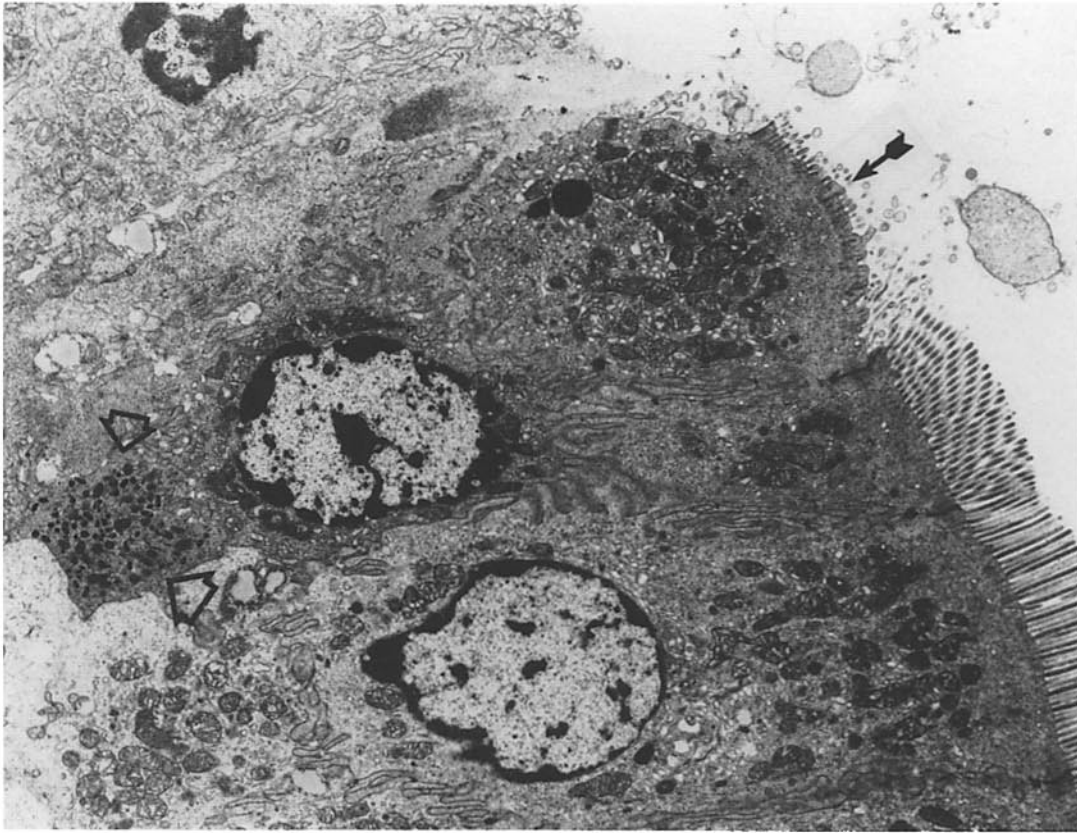

Figure 5. Defective intestinal secretory responses to CT by WW^v mice that have been reconstituted with mast cells. WW^v mice (++ → WW^v) and control littermates (++ → ++) were transplanted with ++ bone marrow as described in Materials and Methods. Ligated intestinal loops from transplanted mice ($n = 5$) were challenged with CT and assessed for fluid secretion as described in Fig. 1. Mean fluid secretion is indicated by filled circles and was as follows: ++ → ++ = 139 ± 8 and ++ → WW^v = 0 ± 0. Fluid secretory responses between the transplanted mouse strains were statistically different at $P = 0.0001$.

by Northern blot analysis. Ligated intestinal loops from C57Bl/6 mice were challenged with CT, and after 2 h, total RNA was extracted. As seen in Fig. 8, CT-challenged intestinal loops contained elevated levels of SCF mRNA (Fig. 8, lanes 3 and 4). Also illustrated in Fig. 8 is the fact that low levels of SCF mRNA are present in control intestinal loops. This was not due to surgery or ligation because low levels of mRNA for SCF was detected by Northern blot in fresh, non-ligated intestinal tissue (data not presented). Thus, these results suggest that CT induces an enhanced production of SCF by the intestinal tract. To explore what cell types might be involved in this enhanced production of SCF, we investigated MODE-K cells which are a mouse intestinal epithelial cell line. MODE-K cells were exposed to CT (100 ng/ml) in vitro and RNA obtained at 2 h. Northern blot analysis of this RNA (Fig. 9), showed that CT enhanced the production of SCF mRNA in MODE-K cells. These results suggest that exposure of the intestinal tract to CT results in the enhanced production of SCF by intestinal epithelial cells.

Fluid from CT-challenged Intestinal Loops Contains SCF. Since increased levels of SCF mRNA were detected in CT-challenged intestinal loops, we initiated experiments to investigate whether fluid secreted into these loops contained SCF. Because there is currently no available ELISA for detecting mouse SCF, we developed a dot-blotting assay using biotinylated rabbit antibody to SCF and an ECL detec-
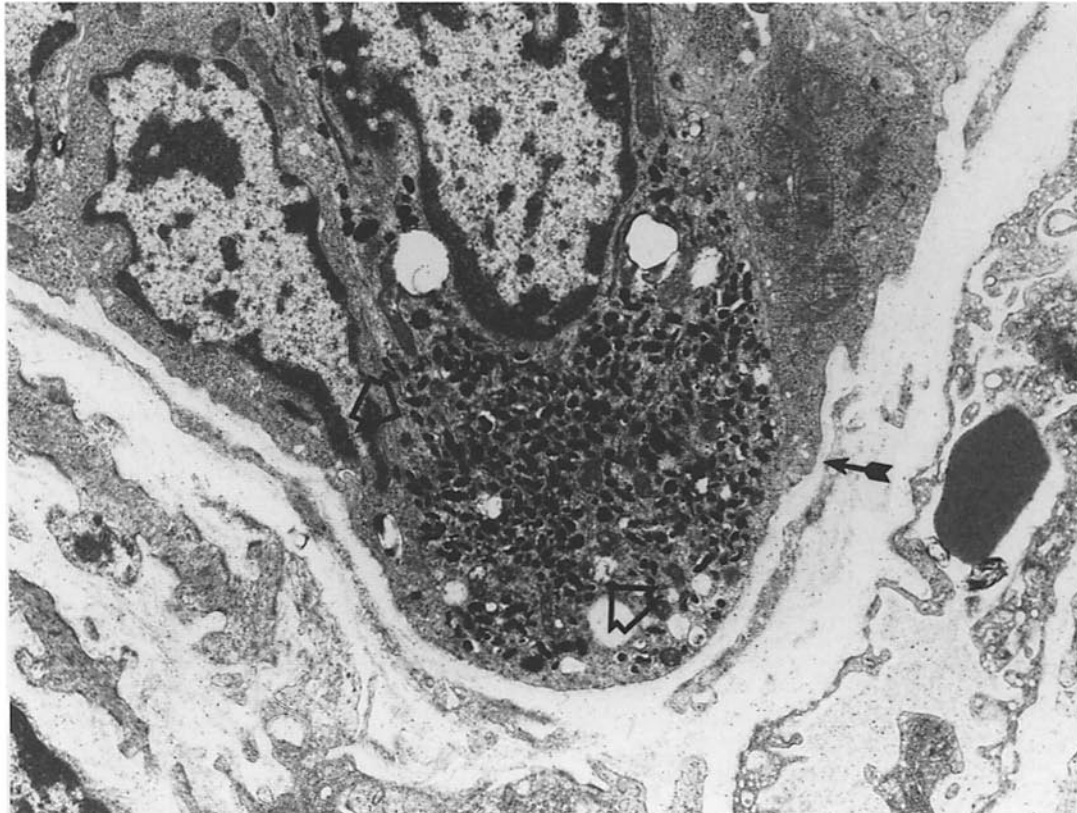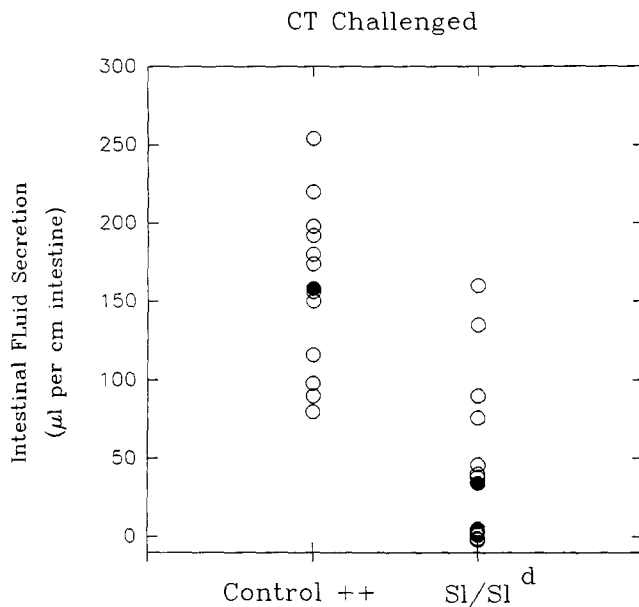
CT Challenged

Figure 7. Defective intestinal secretory responses to CT by Sl/Sl^d mice. Ligated intestinal loops from Sl/Sl^d and control littermates (+ +) were challenged with CT and assessed for fluid secretion at 6–7 h after surgery as presented in Materials and Methods. Fluid secretion from 12 control littermates and 17 Sl/Sl^d mice is presented. Mean fluid secretion is indicated by the filled circles and was as follows: Sl/Sl^d = 34 ± 51 and + + = 158 ± 52. Fluid secretory responses between the mouse strains were statistically different at P = <0.006.

Figure 9. Increased levels of mRNA for SCF in MODE-K cells after exposure to CT. Northern blot analysis of RNA obtained from MODE-K cells at 2 h after exposure to medium only (lane *1*) or CT (100 ng/ml) (lane *2*). Blots were hybridized with a cDNA probe for SCF (*A*), or for G3PDH (*B*).
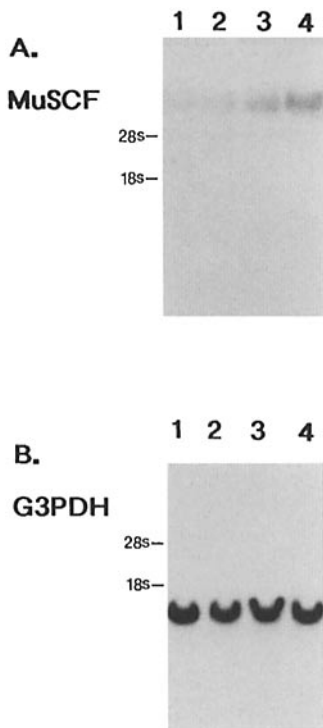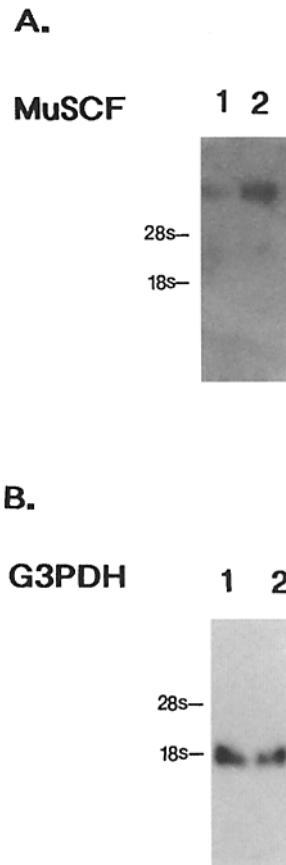

Figure 8. Increased levels of SCF mRNA in CT-challenged ligated loops. Total RNA was obtained from ligated intestinal loops challenged with CT as described in the Materials and Methods. RNA was obtained at 2 h after challenge from control (PBS injected) intestinal loops (lanes *1* and *2*) and from two CT-challenged loops (lanes *3* and *4*). Northern blot analysis was carried out with a cDNA probe for SCF (MuSCF) (*A*) or for G3PDH (*B*).

tion system. Using this assay, we could easily detect 10 ng of SCF (Fig. 10 *A*). We then assessed fluids from CT-challenged intestinal loops for the presence of SCF using this assay. Control loops were gavaged with a volume of PBS that was equivalent to the volume of fluid contained in CT-challenged loops, and this fluid also was assayed for the presence of SCF. As seen in Fig. 10 (*B*, lanes *1–3*) fluid from three different CT-challenged intestinal loops, obtained from Bl/6 mice, contained significant levels of SCF. Fluid obtained from control loops obtained from C57 Bl6 mice, appeared to contain only low levels of SCF (Fig. 10 *B*, lanes *3* and *4*). Sl/Sl^d mice can produce and secrete SCF. However, a quantitative comparison between the levels of SCF produced by Sl/Sl^d versus their littermate controls has not been determined. Since a few of the Sl/Sl^d mice challenged with CT did give a low but detectable fluid response, we investigated whether the intestinal fluid obtained from these mice also contained elevated levels of SCF. As seen in Fig. 10 *C*, fluid obtained from CT-challenged intestinal loops from Sl/Sl^d mice contained only very low levels of SCF. SCF levels appeared to be slightly higher in CT-challenged loops when compared to control loops of Sl/Sl^d mice. This conclusion, however, will have

Figure 6. WW^v mice have a normal pattern of ECC in the small intestine. Tissue sections obtained from WW^v and + + control littermates were assessed by election microscopy for the presence of ECC. *A* (from + +) and *B* (from WW^v) are representative pictures of ECC. Solid arrows identify ECC and open arrows identify granules present in ECC.

A

Concentration of SCF

100 ng 50 ng 25 ng 10 ng 1 ng 0.5 ng 0

B

CT-Challenged   Control

1   2   3   4   5

C

CT-Challenged   Control

1   2   3   4   5   6
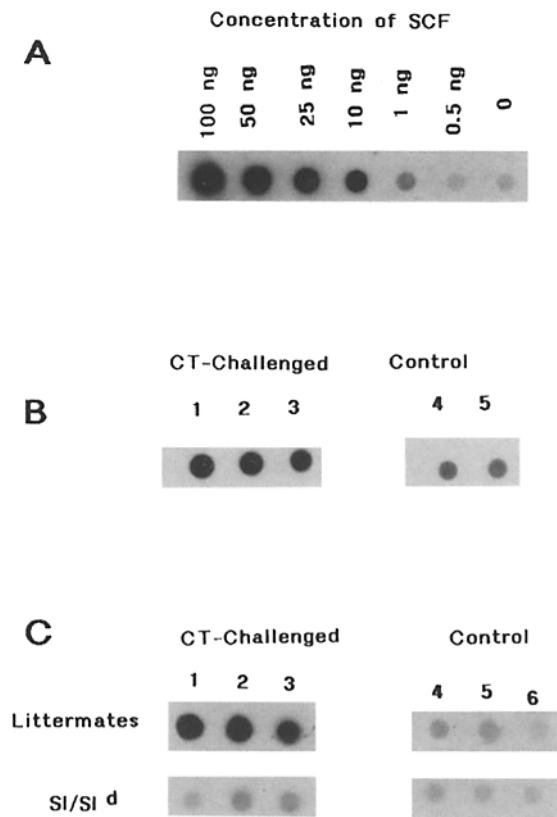
Littermates

Sl/Sl^d

Figure 10. Fluid obtained from CT-challenged intestinal loops contains SCF. Ligated intestinal loops from C57BL/6, Sl/Sl^d, and control littermate mice were challenged with CT or PBS and fluid obtained at 6 h after surgery. Fluid obtained from CT versus PBS-challenged loops were assessed for SCF by dot-blot analysis as described in Materials and Methods. (A) Aliquots of PBS containing FCS (2 μg) and spiked with different concentrations of rSCF were run as a control: lane 1, 100 ng; lane 2, 50 ng; lane 3, 25 ng; lane 4, 10 ng; lane 5, 1 ng; lane 6, 0.5 ng; and lane 7, no SCF added. (B) Intestinal fluid from three different CT-challenged intestinal loops from C57Bl/6 mice were assessed in lanes 1–3. PBS gavaged control intestinal loops were assessed in lanes 3 and 4. (C) Intestinal fluid was assessed from three different Sl/Sl^d mice and from three of their control littermates. SCF levels in fluid from CT-challenged intestinal loops are presented in lanes 1–3, while SCF present in PBS gavaged control intestinal loops are presented in lanes 4–6. Protein levels present in fluids assessed were normalized to 20 μg/ml and 100 μl of fluid blotted in each lane.

to be verified using a more quantitative assay for measuring SCF. In contrast, fluid from CT-challenged intestinal loops obtained from littermate controls contained significantly higher levels of SCF than control loops. Although we cannot quantitate the levels of SCF present in CT versus control loops, data presented in Fig. 10 clearly show that fluid from CT-challenged loops obtained from C57/Bl/6 mice or littermate control mice contained significantly higher levels of SCF than that found in control loops. These results strongly suggest that exposure of the intestinal tract to CT results in enhanced production and secretion of SCF.

CT Induces Increased c-kit Expression on MODE-K Cells. We next investigated whether CT could also upregulate c-kit expression on intestinal epithelial cells. To address this issue, we investigated c-kit expression on MODE-K cells by
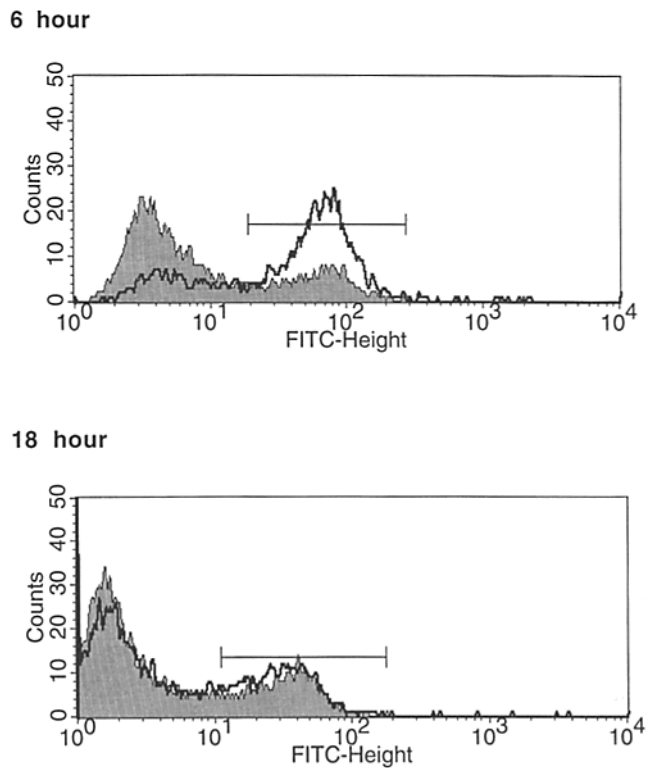
6 hour

18 hour

Figure 11. CT-induced expression of c-kit on MODE-K cells. MODE-K cells were assessed for c-kit expression at varying times of culture with medium versus CT. Flow cytometry histograms are presented for cells obtained at 6 and 18 h of culture. The shaded histogram represents c-kit–positive cells in medium control cultures. The white or open histogram represents the c-kit–positive cells in cultures exposed to CT.

flow cytometry. MODE-K cells were incubated with CT (100 ng/ml) or medium and assessed at varying times for c-kit expression. C-kit was expressed on 25–28% of the MODE-K cells that were maintained in culture medium for 18 h. This percentage of c-kit–positive cells remain constant throughout the 18 h of culture. The percentage of cells expressing c-kit was dramatically increased after exposure to CT. Optimal upregulation of c-kit was observed at 6 h where 74–80% of the MODE-K cells were positive for c-kit (Fig. 11). Interestingly, c-kit expression decreased with increasing time of exposure to CT. After 18 h of culture with CT, c-kit was detected on 37% of the MODE-K cells. These results indicate that CT can upregulate c-kit expression on MODE-K cells and that this upregulation may also occur in vivo and play a role in the intestinal secretory response.

Discussion

In this report we present evidence that SCF–c-kit interactions are important for the intestinal secretory response to CT. The following observations were made in this study: (a) Mice that have mutations at the Sl or W loci have altered intestinal secretory responses to CT, with mutations in the W locus (WW^v mice) resulting in the most dramatic

effects; (b) the CT unresponsiveness of WW^v mice was not due to the absence of mast cells or ECC; (c) intestinal secretory responses to STa are normal in mice that have mutations at the W locus which indicates that CT and STa mediate intestinal secretory responses by different mechanisms; (d) CT can induce an enhanced production of SCF mRNA in the intestinal tract and in an intestinal epithelial cell line; (e) Intestinal fluid present in CT-challenged loops contains elevated levels of SCF; and (f) CT can upregulate c-kit expression on MODE-K cells.

The exact role of c-kit and/or SCF in CT-induced intestinal fluid secretion is not known. These proteins have been shown to play important roles in many diverse biological systems. C-kit–SCF interactions are important for hematopoiesis, mast cell function, melanocyte development, germ cell development, and for the development of the nervous system (18). There is evidence that these proteins may also play a role in nerve cell function (22, 23). B cell and T cell precursors have been shown to express c-kit (46, 47), and recently, TcRγδ$^+$ intraepithelial lymphocytes have been shown to be regulated by SCF–c-kit interactions (48). Relevant to our study are the recent reports that the development of ICC in the intestinal tract is dependent on SCF–c-kit interactions and that these cells mediate intestinal pacemaker activity (19–21). Our findings that WW^v and Sl/Sl^d mice have altered intestinal secretory responses to CT are suggestive evidence that ICC maybe involved in this response. This possibility was especially attractive since CT-induced intestinal fluid secretion has been linked to the enteric nervous system (10, 11). However, in our study, Sl/Sl^d mice appeared to differ from WW^v mice in their intestinal secretory response to CT. CT induced low but detectable intestinal secretory responses in 41% of the Sl/Sl^d mice tested (7 of 17). In WW^v mice, CT induced a low secretory response in 1 of 14 mice investigated (7%). These results are important since Sl/Sl^d and WW^v mice were both reported by Hulzina et al. (19) to lack ICC and pacemaker activity. The fact that WW^v mice differed from the Sl/Sl^d mice with regard to CT-induced fluid secretion suggests that a lack of ICC is not the basis for these aberrant responses to CT. However, until a more definitive study compares the ICC and intestinal pacemaker activity of WW^v versus Sl/Sl^d mice, this question must remain open.

WW^v and Sl/Sl^d mice are virtually devoid of mature, morphologically identifiable mast cells in all organs (18). However, the mast cell deficiency of WW^v mice can be repaired by intravenous injection of normal bone marrow derived from littermate control (+/+) mice (27, 40). In our studies, a lack of mast cells did not appear to be the cause of CT unresponsiveness in WW^v mice. WW^v mice that received a bone marrow transplant from their control littermates had normal numbers of intestinal mast cells, yet were still completely unresponsive to CT. It should be noted that WW^v mice have aberrant secretory responses to antigen-induced intestinal anaphylaxis and that this defect can be corrected by bone marrow transplantation (27).

Enterochromaffin cells (ECC) have been proposed as an important endocrine cell that mediates CT- and STa-induced fluid secretion by the release of their 5-HT and peptides into the lamina propria to activate the myenteric plexus (6, 7, 49). Using electron microscopy, we determined that WW^v mice had a typical intestinal morphology and that they appeared to contain ECC at frequencies identical to their littermate controls (+ +). These results suggested that mutations in the W locus did not alter ECC development. We cannot rule out the possibility that mutations in the W locus could result in alterations in ECC function. However, if ECC are important for STa-induced fluid secretion, then ECC must have at least these functions since WW^v mice were fully responsive to STa-induced fluid secretion.

Although the enteric nervous system has been linked to both CT- and STa-induced fluid secretion (6, 7, 10), these enterotoxins appear to interact with the enteric nervous system via different pathways. CT induces increased cAMP while STa induces increased cGMP (3, 4, 36–38). Neurotensin appears to be involved in CT-induced fluid secretion, but not in STa-induced fluid secretion (8). Our results further strengthen the hypothesis that CT and STa mediate fluid secretion via different mechanisms. SCF–c-kit interactions appear not to be necessary for STa-induced intestinal fluid secretion. These results suggest that STa-induced intestinal secretory responses might not require the ICC network. The abnormal intestinal secretory responses of WW^v and Sl/Sl^d mice to CT indicate that SCF–c-kit interactions are an important component of the CT-induced intestinal response and that these mice may represent a valuable tool for investigating the differences between these two enterotoxins.

The basis for the different intestinal responses to CT in Sl/Sl^d versus WW^v mice is unclear. SCF functions may depend upon whether a c-kit–bearing cell encounters the transmembrane forms of SCF, soluble forms of SCF in solution, or soluble forms sequestered among extracellular matrix proteins (43). In Sl/Sl^d mice there is an intragenic insertion/deletion that removes 242 bp at the 3' end of the coding sequence. This transcript encodes almost all of the extracellular domain of SCF, but lacks coding sequences for both the transmembrane and the intracellular domains of the wild type protein (43–45). It is not clear to what extent soluble SCF is produced by Sl/Sl^d mice. However, it appears that the phenotypic abnormalities of these mice are due to a lack of membrane-associated SCF. Toksoz et al. (50) have shown that the membrane-associated form of SCF plays a critical role in hematopoiesis. In fact, in many circumstances, biologically important effects of SCF are mediated through membrane-associated forms of this molecule (18, 45, 51). One possible explanation for the differential responsiveness of Sl/Sl^d versus WW^v is that CT-induced intestinal secretory responses require c-kit–SCF interactions that involve both soluble and membrane-associated SCF. In WW^v mice this interaction is more dramatically altered because of the aberrant c-kit protein. In Sl/Sl^d mice one component of this response (secreted SCF) is partially functional. In fact, results from our studies suggest that Sl/Sl^d

mice also have a reduced capacity to secrete SCF (Fig. 10 C). This possibility is currently under investigation and will require more definitive measurements of SCF production and secretion. The fact that Sl/Sl^d mice have functional c-kit allows one to investigate whether supplying SCF to these mice could reconstitute the intestinal secretory response to CT. In preliminary studies, we have given Sl/Sl^d mice high doses of recombinant SCF and PEG-SCF (100–500 μg/kg) via intravenous and intraluminal injection and have not been able to alter the CT-unresponsiveness of the intestinal secretory response. However, these negative results are difficult to interpret since we do not know the tissue concentration of SCF present in the intestinal tract of these mice.

The only known mechanism by which SCF exerts biological activity is through its interactions with the c-kit receptor. Thus, intestinal epithelial cells and/or intestinal nerve cells could represent potential targets for SCF action in CT-induced intestinal secretory responses. However, another

possibility that must be considered is the upregulation of c-kit by CT on intestinal cell populations that would normally be c-kit negative. C-kit expression on MODE-K cells, a mouse intestinal epithelial cell line, is significantly upregulated after exposure to CT. While only suggestive, these results may indicate that CT can also upregulate c-kit expression in vivo on different intestinal cell populations.

In summary, our results suggest that CT-induced intestinal secretory responses involve the local production of SCF which, along with other mediators, is required for initiating the secretory response. This hypothesis is supported by the following: (a) WW^v and Sl/Sl^d mice have altered intestinal secretory responses to CT; (b) CT induced elevated levels of SCF mRNA in ligated intestinal loops and in the MODE-K cell line; and (c) SCF could be detected in fluid obtained from CT-challenged intestinal loops. In conclusion, our findings clearly identify SCF as a potentially important factor for the intestinal secretory response induced by CT.

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