Substance P Expression Correlates with Severity of Diarrhea in Cryptosporidiosis

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Cryptosporidiosis, caused by Cryptosporidium parvum, is self-limited in immunocompetent hosts but may cause chronic diarrhea in patients with acquired immunodeficiency syndrome (AIDS). Substance P (SP), a neuropeptide belonging to the tachykinin family, is expressed in gastrointestinal tract and can cause electrogenic chloride anion secretion. Therefore, we studied SP mRNA and protein expression in jejunal tissue samples of patients with AIDS with naturally occurring chronic cryptosporidiosis and healthy volunteers with mild cryptosporidiosis or asymptomatic infection after experimental C. parvum challenge. SP mRNA was associated with symptoms in cryptosporidiosis. SP protein levels were greater in symptomatic than asymptomatic volunteers. Similarly, greater expression of SP mRNA and protein were noted in patients with AIDS with chronic cryptosporidiosis versus immunocompetent volunteers with self-limited infection. This study demonstrates a direct correlation between SP levels and disease severity and may imply that SP plays a role in diarrhea mediation.

Cryptosporidiosis is an important cause of waterborne outbreaks of acute diarrheal illness, childhood diarrhea in developing countries, and AIDS-related diarrhea. Cryptosporidiosis is self-limited in immunocompetent hosts. In patients with AIDS, the disease may cause a life-threatening chronic diarrheal illness that leads to wasting and death. Highly active antiretroviral therapy leads to resolution of symptoms and suppression or elimination of parasite in many, but not all, human immunodeficiency virus (HIV)–infected patients [1–3]. No safe and consistently effective antiparasitic treatment is available for cryptosporidiosis associated with advanced AIDS [4].

Cryptosporidium parvum infection is characterized by intestinal villous damage, reduced sodium absorption, increased electrogenic chloride anion (Cl\textsuperscript–) secretion, and disruption of the epithelial barrier [5–8]. These physiologic alterations lead to watery diarrhea. Proinflammatory cytokines have been hypothesized to mediate these alterations [8, 9]. However, we noted no association between expression of either TNF-α or IL-1β and symptoms of cryptosporidiosis [10, 11].

Neuropeptides form part of the brain-gut axis and are known to mediate many of the inflammatory processes in the intestines. Substance P (SP), a neuropeptide belonging to the tachykinin family, is involved in pain transmission. SP is distributed widely in the gastrointestinal mucosa of healthy human subjects [12–14]. Human monocytes, macrophages, lymphocytes, dendritic cells, eosinophils, and neutrophils produce SP [13, 15–17]. All these cells are widely distributed in the intestinal mucosa. SP can cause Cl\textsuperscript– ion secretion in human gastrointestinal explants [18]. We hypothesized that SP is up-regulated in the intestines in response to C. parvum infection and that SP mediates secretory diarrhea in cryptosporidiosis. To test this hy-
pothesis, we studied qualitative and semiquantitative expression of SP in jejunal tissue samples from patients with AIDS with chronic cryptosporidiosis (severe disease) and from healthy immunocompetent volunteers challenged with C. parvum (mild disease). Tissue samples obtained via biopsy of healthy immunocompetent volunteers before challenge (negative control subjects) and after asymptomatic challenge served as negative control samples.

**MATERIALS AND METHODS**

*Healthy immunocompetent volunteers.* Tissue samples were available from 24 healthy volunteers (aged 18–50 years who were screened to exclude immune dysfunction) who were challenged with 10–1 × 10⁶ C. parvum oocysts as part of other studies [19–22]. After challenge, all stool samples were collected, and oocyst excretion was measured, as described elsewhere [19–22]. Symptoms, including diarrhea, abdominal pain, nausea, or vomiting, were recorded for 6 weeks after challenge. Jejunal tissue samples from 24 volunteers were collected 3–19 days after challenge via biopsy with upper endoscopy with a pediatric colonoscope. Healthy immunocompetent volunteers developed 2 different responses to C. parvum challenge. Eight volunteers did not develop gastrointestinal symptoms, and 16 volunteers developed mild symptoms. The median number of watery stools passed by symptomatic volunteers was 4.5 during a median duration of 4 days. Control samples included prechallenge tissue samples from 8 normal subjects.

*Patients with AIDS.* Seven patients with AIDS with naturally occurring chronic cryptosporidiosis also underwent jejunal endoscopy and biopsy. All had chronic watery diarrhea for several months.

**Cytokine cDNA, plasmids, and preparation of [³⁵S]-labeled riboprobes.** Plasmid pBluescript SK⁺ containing cDNA for human SP precursor (preprotachykinin; ATCC) were prepared by ion-exchange chromatography (Qiagen) [23, 24]. After linearization of the plasmid cDNA with appropriate enzymes (antisense, EcoRI; sense, NotI), labeled probes were synthesized by in vitro transcription in the presence of 250 µCi of [³⁵S]-UTP by T3 polymerase (to generate antisense probe) or T7 polymerase (to generate sense negative control probe) with a commercially available kit (Amersham Life Sciences). The template was digested with RNase-free DNase, the [³⁵S]-UTP-labeled probe was precipitated with 100% ethanol and 1 mol ammonium acetate salt, washed with 70% ethanol, and suspended in dithiothreitol (DTT).

**In situ hybridization.** Paraffin-embedded jejunal tissue sample sections were treated with xylene to remove the paraffin, rehydrated with decreasing concentrations of ethanol (90%–70%), and incubated with prehybridization buffer for 1 h. The tissue samples were then hybridized with [³⁵S]-UTP-labeled riboprobes (in hybridization solution) for 3 h, as described elsewhere [11, 24–26]. After hybridization, the slides were washed twice with 2× standard saline citrate (SSC), incubated with 50% formamide, washed with decreasing concentrations of 2× SSC, and digested with RNase for 30 min at 37°C to remove nonhybridized probe. The slides then were immersed in autoradiographic emulsion (Eastman Kodak; for 48 h at 24°C) developed with photographic developer. After fixation, the slides were counterstained with Giemsa stain. The sense-strand probe was used as a negative control for each volunteer.

To quantitate and compare the signal intensity between the patients with AIDS and immunocompetent volunteers with cryptosporidiosis, the slides were examined by bright field microscopy, and the number of cells overlaid with numerous silver granules was counted. Slides with signal above background were graded as 1+ (1–2 positive cells/section), 2+ (>2 positive cells/section but <1/low-power field [×20 lens]), 3+ (1 positive cell/low-power field but <1/high-power field [×40 lens]), or 4+ (≥1 positive cells/high-power field).

**Immunohistochemistry.** To determine the expression of SP protein, immunoperoxidase stains were performed on 5-µm–thick, paraformaldehyde-fixed jejunal sections. Immunohistochemistry was performed by the avidin–biotin method [11, 24–26] via an automated immunostainer (Biogenex), and polyclonal rabbit antibody to human SP (1:20; Chemicon). In brief, paraffin-embedded jejunal sections were treated with xylene, to remove the paraffin, rehydrated with decreasing concentrations of ethanol (90%–70%), and washed with distilled water. The endogenous peroxidase activity was quenched by treatment of the tissue for 15 min with 3% hydrogen peroxide, after which, the slides were washed with PBS.

To reduce nonspecific binding of the primary antibody with the tissues, before primary antibody treatment, the tissues were first blocked for 30 min at room temperature (RT) with CAS Block from Zymed Laboratories. The CAS block was drained off, and the tissues were treated with 1:20 dilution of polyclonal rabbit antibody to human SP for 30 min at RT (Chemicon). The slides then were washed thoroughly with 1× PBS (3 times; each wash for 3 min at RT) and treated with goat anti–rabbit biotinylated conjugate (1:100 in PBS; 30 min at RT). The slides were washed with 1× PBS (3 times; each wash for 3 min at RT), and tissues were treated with 2 drops of avidin–biotin complex (Vector) for 30 min at RT, followed by a thorough washing with 1× PBS (3 times; each wash for 3 min at RT). Finally, the tissues were stained for 30 s with freshly prepared solution of DAB (3,3’-diamino benzidine), according to the instructions outlined in the kit (Vector). The slides were finally rinsed in running water, dried, and observed via light microscopy, as described elsewhere [23]. Slides were considered to be positive if brown staining was noted within the cytoplasm of
Figure 1. Expression of substance P precursor (SP) mRNA in cryptosporidiosis. SP precursor mRNA expression was studied by in situ hybridization (using $[^{35}S]$-labeled riboprobes) in paraformaldehyde-fixed, paraffin-embedded jejunal tissue samples derived from a healthy volunteer (A; original magnification, ×200); healthy volunteer who did not develop symptoms after experimental Cryptosporidium parvum challenge (B; original magnification, ×200); healthy volunteer who developed self-limited cryptosporidiosis after experimental C. parvum challenge showing 2 positive cells in the villi expressing SP precursor mRNA (arrows) (C; original magnification, ×200); and a patient with AIDS with naturally occurring chronic cryptosporidiosis showing numerous positive cells expressing SP precursor mRNA in the lamina propria and crypts (D; original magnification, ×100).

cells above the level of nonspecific signal in tissue cells. Positive slides were graded as 1+ to 4+, as described above.

These studies were approved by the committee for the protection of human subjects at the University of Texas at Houston and the Institutional Review Board for Human Subjects Research at Baylor College of Medicine.

RESULTS

SP mRNA was not detected in any of the 8 prechallenge jejunal tissue samples (figure 1A). Similarly, none of the 8 volunteers who remained asymptomatic after experimental C. parvum challenge had detectable SP mRNA (figure 1B). Faint diffuse signal for SP protein was detected by immunohistochemistry in the tissue samples obtained from the prechallenge biopsies performed ($n = 7$) and the asymptomatic volunteers ($n = 7$; figure 2A and 2B).

Increased SP mRNA and protein were detected in postchallenge tissue samples from volunteers with symptomatic cryptosporidiosis. In these individuals, SP mRNA was detected as numerous black silver grains overlying cells in the epithelium of villi (figure 1C). SP protein was predominantly detected in the villus epithelium (data not shown) and cells in the lamina propria (figure 2C).

SP mRNA was more frequently expressed in volunteers with symptomatic cryptosporidiosis, compared with that in asymptomatic volunteers ($7/16$ symptomatic vs. $0/8$ asymptomatic volunteers; $P \leq .05$, Fisher’s exact test; figure 3). The level of SP protein by immunohistochemical stains was significantly greater in volunteers with symptomatic cryptosporidiosis, compared with that in the C. parvum–challenged healthy volunteers who did not develop symptoms. The median level of SP protein in volunteers with symptomatic cryptosporidiosis was 2+, with the values ranging from 1 to 3, whereas the median value in C. parvum–challenged healthy volunteers who did not develop symptoms was 1+, with the values ranging from 1+ to 2+ ($P \leq .01$, Wilcoxon rank-sum test).

Jejunal tissue samples from all 7 patients with AIDS with chronic cryptosporidiosis expressed SP mRNA and protein (figures 1D, 2D, and 3). In the patients with AIDS, SP mRNA and
Figure 2.  Expression of substance P (SP) protein in cryptosporidiosis. SP protein expression was studied by immunohistochemistry via a polyclonal antibody to SP in paraformaldehyde-fixed, paraffin-embedded jejunal tissue samples derived from a healthy volunteer (A; original magnification, ×200); a healthy volunteer who did not develop symptoms after experimental Cryptosporidium parvum challenge (B; original magnification, ×200); a healthy volunteer who developed self-limited cryptosporidiosis after experimental C. parvum challenge (C; original magnification, ×200); and a patient with AIDS with naturally occurring chronic cryptosporidiosis (D; original magnification, ×100).

protein were detected in the lamina propria and crypt epithelium (figures 1D and 2D). The proportion of patients with AIDS with chronic cryptosporidiosis expressing SP mRNA was significantly higher, compared with that in symptomatic volunteers (7/7 vs. 7/16; P = .02, Fisher's exact test; figure 3). The semiquantitative signal strength of SP precursor mRNA and protein expression was stronger in patients with AIDS, compared with that in immunocompetent volunteers with mild, self-limited cryptosporidiosis (P ≤ .01, Wilcoxon rank-sum test; figure 4). The level of SP mRNA expression in patients with AIDS with severe cryptosporidiosis ranged from 3+ to 4+, with a median value of 4+, whereas, in immunocompetent volunteers with mild, self-limited cryptosporidiosis, values ranged from 0 to 2+, with a median value of 0 (P ≤ .01, Wilcoxon rank-sum test; figure 4). Similarly, the median level of SP protein in the patients with AIDS with chronic cryptosporidiosis was 4+, with the values ranging from 2+ to 4+, whereas the median value in the immunocompetent volunteers with mild, self-limited cryptosporidiosis was 2+, with the values ranging from 1+ to 3+ (P ≤ .01, Wilcoxon rank-sum test).

DISCUSSION

SP mRNA and protein expression was detected in gut tissue samples from immunocompetent volunteers with mild, self-limited experimental cryptosporidiosis. SP mRNA was not detected in prechallenge jejunal tissue samples or in tissue samples from healthy immunocompetent volunteers who did not develop symptoms after C. parvum challenge. Low levels of SP protein were detected in both groups, which is consistent with the findings of other groups, in which low-level expression of SP protein in normal intestinal samples was detected [13, 14]. Both the frequency and intensity of SP expression in patients with AIDS with chronic cryptosporidiosis was significantly greater than in immunocompetent volunteers with self-limited cryptosporidiosis. Similarly, expression was greater in symptomatic than asymptomatic volunteers. Thus, both the frequency and intensity of SP expression correlated with severity of illness.

The elevated levels of SP might be the result of the infection and not the cause of the symptoms associated with the illness. SP could be elevated as a result of the influx of inflammatory
Figure 3. Correlation of substance P (SP) mRNA with symptoms in cryptosporidiosis. Expression of SP precursor mRNA was associated with symptoms in cryptosporidiosis. Intestinal tissue samples from patients with AIDS with cryptosporidiosis and prechallenge and postchallenge tissue samples from immunocompetent volunteers with experimental Cryptosporidium parvum infection were examined by in situ hybridization for SP precursor mRNA. The percentage of subjects with SP precursor mRNA was compared in groups, including volunteers before C. parvum challenge, volunteers with asymptomatic or mild, self-limited cryptosporidiosis, and patients with AIDS with severe cryptosporidiosis. SP precursor mRNA was more frequently detected in immunocompetent volunteers with symptomatic vs. asymptomatic infection (*, Fisher’s exact test), and SP precursor mRNA was more frequently detected in patients with AIDS with chronic cryptosporidiosis vs. immunocompetent volunteers with mild symptomatic infection (**, Fisher’s exact test).

Figure 4. Levels of substance P (SP) precursor expression during active cryptosporidiosis. Levels of SP mRNA expression, as graded semiquantitatively by light microscopy (after in situ hybridization), in patients with AIDS vs. immunocompetent volunteers with mild, self-limited cryptosporidiosis. Levels of SP mRNA expression in 7 patients with AIDS vs. 16 immunocompetent volunteers with symptomatic cryptosporidiosis (*P < .001, Wilcoxon rank-sum test).
suggests the involvement of SP in inflammatory bowel disease [27, 32–36]. In animal models, SP antagonist (SR140333) can modify the course of experimental colitis induced in the rat by trinitrobenzene sulfonic acid and reduce the severity of colitis, as well as alterations in contractility [31].

There are limited data on SP or its receptor in diarrhea associated with cryptosporidiosis. One study noted increased expression of the SP receptor NK1-R in mice infected with *C. parvum* [37]. However, murine cryptosporidiosis is not associated with diarrhea, and that study did not assess GI function or symptoms.

SP also mediates inflammatory processes in other enteric infections. *Clostridium difficile* toxin A induces acute inflammation of the intestine initiated by release of SP and activation of the NK1-R [38–40]. SP antagonist inhibits rat intestinal responses to *C. difficile* toxin A. Inhibition was dose dependent and caused a significant reduction of inflammation within the lamina propria, a reduction in the necrosis of intestinal epithelial cells, and the complete inhibition of *C. difficile* toxin A–mediated release of rat mast cell protease 2, a specific product of rat mucosal mast cells [41]. Cholera toxin–induced water and electrolyte secretion were inhibited by antagonists to SP [42].

In conclusion, our studies noted significant association of SP with symptoms in human cryptosporidiosis. The level of SP correlated with disease severity. Taken together with the current background information on SP, our data may suggest a role of SP in diarrhea mediation in human cryptosporidiosis. However, further studies are required to definitely prove that SP is responsible for inducing *C. parvum*–induced physiologic alterations that lead to the pathogenesis of the disease.

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**References**