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Enhanced intestinal motor response to cholecystokinin in post-*Nippostrongylus brasiliensis*-infected rats: modulation by CCK receptors and the vagus nerve

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Abstract *The jejunal inflammation induced in rats by the nematode Nippostrongylus brasiliensis is followed by intestinal neuroimmune alterations including mast cell hyperplasia and nerve remodelling. On the other hand, cholecystokinin (CCK) plays a pivotal role in the regulation of intestinal motility. The aim of this study was to determine whether the intestinal motor response to CCK is altered 30 days after infection by N. brasiliensis. Thus, CCK-8 (50 μg kg^{-1} intraperitoneally) disrupted the pattern of jejunal migrating myoelectric complexes for a longer time in postinfected rats (95.5 ± 3.5 min) than in controls (48.1 ± 5.1 min). This enhanced jejunal response was also found after oral administration of the potent releaser of endogenous CCK, soybean trypsin inhibitor. In contrast, no alteration of the inhibition of colonic motility by CCK administration was observed. The increased responsiveness of jejunal motility to CCK persisted after mast cell stabilisation or depletion but was prevented by atropine, devazepide and* L*-365260 (CCK-A and CCK-B receptor antagonists, respectively) and vagotomy. These results indicate that neuroimmune alterations after N. brasiliensis infection lead to an increased intestinal motility response to CCK that involves a cholinergic mediation, a vagal pathway and alterations in intestinal CCK-A and CCK-B receptors.*

Keywords *CCK, CCK receptors, intestinal motility, nematode postinfection, rat, vagus nerve.*

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INTRODUCTION

Severe intestinal infections are often followed by symptoms of irritable bowel syndrome (IBS),[1] characterized by abnormalities of intestinal motility and visceral hypersensitivity to various stimuli.[2] There is growing evidence that these alterations in both motility and sensitivity involve neuroimmune modifications of the intestinal mucosa.[3,4] An increase in mast cell numbers has been described in the intestinal mucosa of IBS patients[5–7] and because of the close relationships between mast cells and sensory nerve fibres,[8] it has been suggested that mast cells have a major role, which would explain the motor and sensitive alterations observed in IBS patients.[5] On the other hand, cholecystokinin (CCK) plays a pivotal role in the regulation of digestive physiology including intestinal motility.[9,10] Its action on intestinal motility is located at the periphery or at the level of the central nervous system,[11,12] and involves CCK-A and CCK-B receptors,[13,12] cholinergic stimulation,[14] and afferent vagal fibres.[15] There is also evidence for involvement of CCK in the neuroimmune alterations of the intestinal wall. It has been suggested that endogenous CCK degranulates mast cells through the stimulation of CCK-B receptors[16]. On the other hand, CCK induces higher pain scores[17] and an exaggerated motor response of the small intestine[2] when injected into IBS patients compared with controls.

Rats infected with the nematode *Nippostrongylus brasiliensis* provide a well-known model for postinfective neuroimmune alterations of the small intestine. *N. brasiliensis* infection has been shown to induce a long-term mast cell hyperplasia for several weeks after an acute inflammation of the proximal jejunum for 2 weeks.[18–21] The intestinal mastocytosis is found associated with a structural plasticity of the intestinal wall and mucosal nerve remodelling,[22] increased excitability of enteric neurons,[23] and an

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increase in substance P (SP) content in the myenteric plexus[24] and SP-immunoreactive mucosal nerve fibres.[25] Moreover, a close apposition of mast cells and nerves has been described in humans[8] as well as in rats.[26]

It has been shown that the mastocytosis induced by nematode infection is associated with a hypersensitivity to distension limited to the jejunum, in which mast cell degranulation is involved.[27] The postinfective neuroimmune alterations do not induce changes in the basal motor profile of the intestine[28] but are associated with an increased intestinal motor response to acetylcholine.[21]

Taken together, these elements lead us to investigate whether the intestinal motor response to CCK is modified in *N. brasiliensis* postinfected rats. We also investigated the role of mast cell degranulation, CCK-A and CCK-B receptors and vagus nerves in the altered intestinal motor response to CCK in postinfected rats.

MATERIALS AND METHODS

Animals

Experiments were performed in 88 male Wistar rats (Harlan, Ganat, France) initially weighing between 150 and 200 g. They were housed under controlled lighting conditions, with standard diet (A 04: Usine d'Alimentation Rationnelle, Epinay-sur-Orge, France) and water provided *ad libitum*. All protocols were approved by the Local Animal Care and Use Committee of Institut National de la Recherche Agronomique.

Intestinal myoelectric studies

Animals were prepared for long-term recordings of intestinal myoelectric activity with the use of a previously described technique.[29] Briefly, under ketamine anaesthesia 120 mg kg^{-1} intraperitoneally (i.p.) (Imalgene 1000; Mérial, Lyon, France), two groups of three insulated NiCr wire electrodes (80 μm diameter, 80 cm length) were implanted in the wall of the proximal jejunum 5 and 15 cm distal to the ligament of Treitz. In 48 rats, a group of electrodes was also implanted in the proximal colon, 2 cm distal to the caeco-colonic junction. The jejunal sites were chosen according to the observation that *N. brasiliensis* is primarily implanted in the proximal half of the intestine and induces a mastocytosis, which is well established at this level at 30 days postinfection.[30,31] The colonic site was considered as a control because no increase in mast cell numbers has been observed at this level after *N. brasiliensis* infection.[30,31]

Jejunal and colonic myoelectric activities were recorded with an electroencephalograph (Minihuit; Alvar, Paris, France), using a paper speed of 3.6 cm min^{-1} and a time constant of 0.03 s. Jejunal spiking activity collected by bipolar derivation was summed every 20 s by an integrator circuit and automatically plotted on the y-axis of a potentiometric recorder (L 6514; Linseis, Selb, Germany) with a paper speed of 6 cm h^{-1}. This integrated record permitted a clear determination of the cyclic occurrence of migrating myoelectric complexes (MMC) and of the duration of their disruption after administration of CCK.

Subdiaphragmatic vagotomy

In 12 rats, just before implantation of the electrodes, the anterior and posterior trunks of the vagus nerve were transected at 1 cm above the gastro-esophageal junction, as previously described.[31] In 12 other rats, sham vagotomy was performed by a 5 min exteriorisation of the stomach from the abdominal cavity.

Culture and infection techniques

N. brasiliensis was maintained in continuous culture by harvesting worm eggs from infected rats and growing them to the third larval (L3) stage on vermiculite and filter paper, using a technique described by Jennings *et al.*[32] The appropriate rats were infected by subcutaneously injecting 3,000 L3 infective larvae of *N. brasiliensis* in 0.5 mL sterile saline into the rat flank. Controls received sterile saline only.

Experimental design

For each series of experiment, treatments were applied in parallel to control and postinfected rats, then motility parameters were compared between the two animal populations where n represented the number of animals in each group.

All studies were carried out between 30 and 50 days post-*N. brasiliensis* infection.

Soybean trypsin inhibitor (SBTI) and CCK-8 In a first series of experiments, in rats fasted for 14 h ($n = 6$), soybean trypsin inhibitor (SBTI, 1% solution w/v, 1 mL) was administered by oro-gastric gavage (per oral; p.o.) when the myoelectrical activity of the jejunum displayed a MMC pattern. CCK-8 was administered i.p. at doses of 10 ($n = 6$) and 50 μg kg^{-1}. For the dose of 50 μg kg^{-1}, CCK was administered alone ($n = 8$) or after the vehicle (0.2 mL DMSO i.p.; $n = 8$).

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Mast cell stabilization and depletion To assess the role of mast cells in the response to CCK-8 (50 μg kg^{-1} i.p.) in controls and postinfected rats, two groups of rats were pretreated with the mast cell stabilizer, ketotifen (1 mg kg^{-1} i.p.) and two other groups with the mast cell degranulator, BrX 537A (2 mg kg^{-1} i.p.)[33]. Rats ($n = 8$) received two injections of BrX 537A, at 14 h interval, the last one being performed 2 h before the administration of CCK-8. The other rats ($n = 8$) were given ketotifen twice daily for 4 days, and received the last administration 2 h before CCK-8. The dose of 2 mg kg^{-1} BrX 537A was chosen because it was found to reduce the number of intestinal granulated mast cells 1 and 5 h after its administration to rats.[33] The 4-day treatment with ketotifen twice daily at the dose of 1 mg kg^{-1} i.p. has previously been used to prevent mast cell degranulation in rats.[34]

Muscarinic and CCK receptor blockade Atropine (1 mg kg^{-1} i.p.) was injected 30 min before CCK-8 (50 μg kg^{-1} i.p.; $n = 6$). The CCK-A receptor antagonist devazepide and the CCK-B antagonist L-365260 were administered i.p., at doses of 50, 100 and 500 μg kg^{-1}, 45 min before CCK-8 (50 μg kg^{-1} i.p.; $n = 8$). CCK-8 (50 μg kg^{-1} i.p.), preceded by the CCK receptor antagonists (100 μg kg^{-1} i.p.) or their vehicle, was also administered in sham vagotomized ($n = 6$) and vagotomized rats ($n = 6$).

Chemicals

SBTI (type IIS; 1 % solution w/v) was dissolved in distilled water containing carboxymethylcellulose 0.4 % w/v. Sulphated CCK-8, atropine sulphate and ketotifen fumarate were dissolved in sterile saline 0.9 %. BrX 537A (bromolasalocid ethanolate), devazepide and L-365260 were dissolved in dimethylsulphoxide (DMSO). All compounds were obtained from Sigma (St Louis, MO, USA) except BrX 537A, which was a gift from Roche Laboratories (London, UK) and the CCK receptor antagonists, which were a gift from Merck Research Laboratories (Rahway, NJ, USA).

Data analysis

The duration of MMC disruption and the number of colonic contractions obtained after the different treatments were expressed as mean ± SEM. Data from each group of animals were considered as resulting from one experiment and statistical analysis was performed using analysis of variance (ANOVA) followed by the Tukey's *post hoc* test. $P < 0.05$ was considered statistically significant.

RESULTS

Basal conditions

In 14 h-fasted control and postinfected rats, the intestinal motor profile consisted of MMC. The number of MMC for a 2 h period was 6.67 ± 0.21 in controls and 8.67 ± 0.95 ($P > 0.05$) in postinfected rats. These values were not significantly modified after vagotomy in both groups (7.86 ± 0.46 and 7.88 ± 0.48, respectively). At colonic level, the number of spike bursts for a 20 min period was 19.6 ± 1.2 in controls and 20.4 ± 1 in postinfected rats ($P > 0.05$).

CCK-8 and SBTI

In control animals, CCK-8 administered i.p. at doses of 10 and 50 μg kg^{-1} disrupted the MMC pattern for 30.0 ± 5.8 and 48.1 ± 5.1 min respectively. These values were significantly increased in postinfected rats (51.5 ± 4.3 and 95.5 ± 3.5 min, respectively; $P < 0.05$) (Figs. 1A; 2A,B). After administration of the vehicle, 50 μg kg^{-1} CCK-8 i.p. disrupted the MMC pattern for 46.3 ± 4.8 and 99.3 ± 4.6 min in controls and postinfected rats, respectively ($P < 0.05$) (Fig. 1A). At colonic level, 50 μg kg^{-1} CCK-8 i.p. decreased significantly ($P < 0.05$) the number of contractions for the first 20 min both in controls (7.0 ± 0.6) and postinfected rats (6.9 ± 0.6) compared with basal conditions.

Oral administration of SBTI (1% solution w/v; 1 mL) immediately disrupted the MMC pattern which was replaced by a continuous activity for 21.7 ± 4.6 min in controls and 44.7 ± 6 min in postinfected rats ($P < 0.05$) (Fig. 1A). At colonic level, SBTI did not significantly modify the number of spike bursts for the first 20 min both in controls and postinfected rats (11.8 ± 0.9 and 12.1 ± 2.2, respectively, $P > 0.05$) compared with basal conditions.

Mast cell stabilization and depletion

A pretreatment with the mast cell stabilizing agent ketotifen (1 mg kg^{-1}; 4 days, twice daily) did not significantly modify the MMC disruption induced by 50 μg kg^{-1} CCK-8 both in controls and postinfected rats (47.9 ± 4.9 and 89.0 ± 9.5 min, respectively); Figs. 1A, 2C). In the same conditions, previous administration of the mast cell degranulator BrX 537A (2 mg kg^{-1}, twice) did not significantly modify the duration of MMC disruption induced by CCK-8 in both groups (43.6 ± 5.3 and 88.9 ± 3.6 min, respectively; Fig. 1A).

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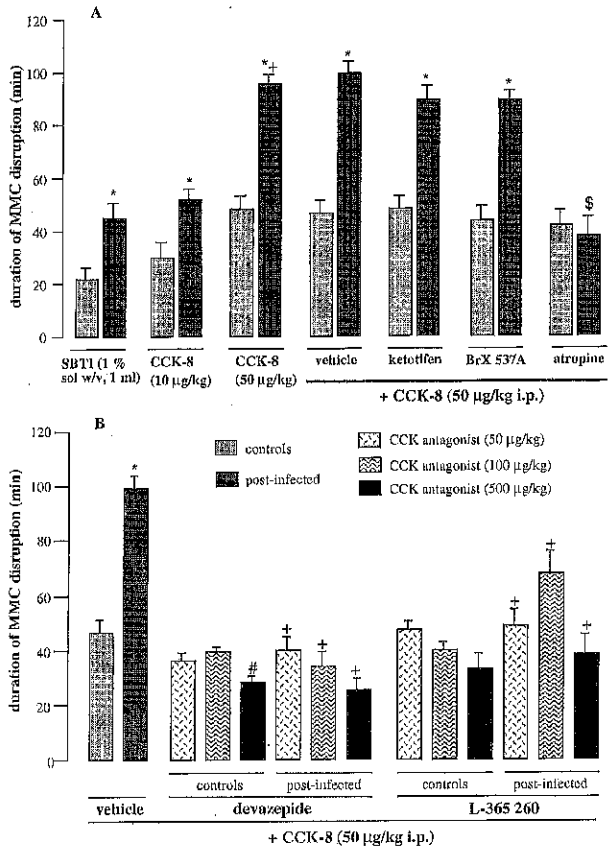

Figure 1 (A) Duration of MMC disruption (min) after SBTI (1% solution w/v, 1 mL; $n = 6$) or CCK-8 (10 μg kg^{-1} and 50 μg kg^{-1} i.p.; $n = 6$ and 8) in controls and postinfected rats, after CCK-8 (50 μg kg^{-1} i.p.) in vehicle-pretreated controls ($n = 8$) and postinfected rats ($n = 8$) or in controls and postinfected rats pretreated with ketotifen (1 mg kg^{-1} i.p., 4 days twice; $n = 8$) or BrX 537A (2 mg kg^{-1} i.p., twice; $n = 8$), after CCK-8 (50 μg kg^{-1} i.p.) in atropine (1 mg kg^{-1} i.p.)-pretreated controls ($n = 6$) and postinfected rats ($n = 6$) (mean ± SEM). *Significantly different from the respective control group ($P < 0.05$); + significantly different from postinfected rats (CCK-8, 10 μg kg^{-1} i.p.; $P < 0.05$) $Significantly different ($P < 0.001$) from vehicle-pretreated postinfected rats. (B) Duration of MMC disruption (min) after CCK-8 (50 μg kg^{-1} i.p.) in vehicle-pretreated controls ($n = 8$) and postinfected rats ($n = 8$) or pretreated-rats with devazepide or L-365260 (50, 100 or 500 μg kg^{-1} i.p., $n = 8$) (mean ± SEM). *Significantly different ($P < 0.001$) from the respective control group; +significantly different ($P < 0.01$) from vehicle-pretreated postinfected rats; #Significantly different ($P < 0.05$) from vehicle-pretreated controls.

Muscarinic and CCK receptor blockade

The muscarinic receptor antagonist, atropine (1 mg kg^{-1}) did not reduce the effect of 50 μg kg^{-1} CCK-8 in controls (41.5 ± 5.9 min) but reduced it significantly

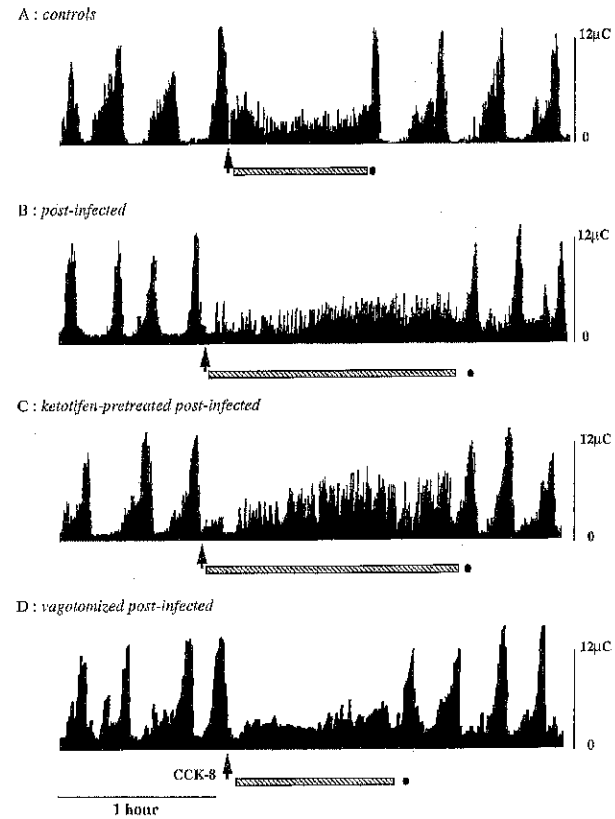

Figure 2 Representative integrated jejunal electromyograms obtained in control and post-*N. brasiliensis*-infected rats after i.p. administration of CCK-8 (50 μg kg^{-1}), in basal conditions (A–B), in postinfected rats pretreated with ketotifen (C) or in vagotomized postinfected rats (D). Arrow, administration of CCK-8; bar, MMC disruption period; point, first phase III after MMC disruption.

($P < 0.001$) in postinfected rats (37.7 ± 7.0 min; Fig. 1A).

The CCK-A receptor antagonist, devazepide, at 50 and 100 μg kg^{-1} did not significantly reduce the duration of MMC disruption induced by 50 μg kg^{-1} CCK-8 in controls (35.9 ± 3.3 and 39.6 ± 1.6 min, respectively, $P > 0.05$) whereas the dose of 500 μg kg^{-1} significantly reduced this response (28.3 ± 2.1 min, $P < 0.05$). In postinfected rats, devazepide at 50, 100 and 500 μg kg^{-1} reduced the response to CCK-8 (40.0 ± 4.4, 33.8 ± 5.7 and 25.0 ± 4.7 min, respectively, $P < 0.001$; Fig. 1B).

The CCK-B receptor antagonist, L-365260, at 50, 100 and 500 μg kg^{-1} did not shorten the MMC disruption induced by CCK-8 in controls (47.3 ± 3, 39.8 ± 3.0 and 32.8 ± 6.0 min, respectively, $P > 0.05$) but reduced the response in postinfected rats (48.6 ± 5.8, $P < 0.001$; 67.8 ± 8.0, $P < 0.01$ and 38.3 ± 7.1 min, $P < 0.001$, respectively) (Fig. 1B).

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Role of the vagus nerve

A bilateral vagotomy did not reduce the response to 50 μg kg^{-1} CCK-8 in controls (50.0 ± 2.7 min, $P > 0.05$) but reduced it in postinfected rats (43.3 ± 7.6 min, $P < 0.001$) compared with sham vagotomized controls and postinfected rats (45.0 ± 0.9 and 88.5 ± 5.6 min, respectively; Figs 3, 2D). In vagotomized control rats, devazepide and L-365260 at the dose of 100 μg kg^{-1} did not significantly reduce the response to CCK-8 (23.3 ± 7.3 min and 43.3 ± 0.8 min, respectively; $P > 0.05$). In vagotomized postinfected rats, devazepide and L-365260 (100 μg kg^{-1}) did not significantly reduce the response to CCK-8 (37.5 ± 3.5 and 38.5 ± 5.0 min, respectively, $P > 0.05$; Fig. 3).

DISCUSSION

Our results indicate that the neuroimmune changes of the intestinal wall which follow the inflammation induced by *N. brasiliensis* are associated, between 30 and 60 days postinfection, with a longer intestinal motor response to both exogenous CCK and substance-releasing endogenous CCK. This increased reactivity to CCK appears to be limited to the jejunum, and to involve CCK-A and CCK-B receptors, the vagus nerve and a cholinergic mechanism.

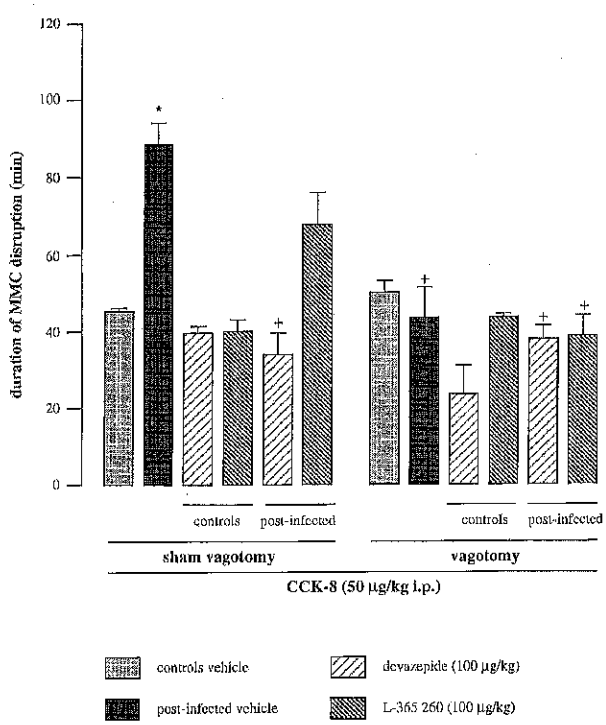

Figure 3 Duration of MMC disruption (min) after CCK-8 (50 μg kg^{-1} i.p.) in sham vagotomized ($n = 6$) and vagotomized controls ($n = 6$) and postinfected rats ($n = 6$) pretreated with vehicle, devazepide or L-365260 (100 μg kg^{-1} i.p.) (mean ± SEM). *Significantly different ($P < 0.001$) from sham vagotomized vehicle-pretreated controls; +significantly different ($P < 0.001$) from sham vagotomized vehicle-pretreated postinfected rats.

In rats, infection with the nematode *N. brasiliensis* is a widely used model for the study of immune reactions of the gut. We have used this model for the study of long-term neuro-immune changes induced by the infection. The parasite develops in the proximal small intestine and produces an early inflammatory phase which peaks at 10–12 days after infection.[28] It also induces an intestinal mast cell hyperplasia[21] and nerve remodelling[35,22] which persist for several weeks after the inflammation has resolved. Changes in gut neurotransmitter concentration have also been identified during *T. spiralis* infection.[36]

It has been previously shown that the basal intestinal motility pattern, with regular occurrence of MMC, is disrupted during the acute phase of inflammation from the 4th day after infection and that a normal pattern is recovered around the 21st day.[28] We confirm that 30 days after infection the intestinal MMC pattern is normal. This indicates that the nerve remodelling observed at this postinfection period does not correspond to profound alterations in myenteric plexus as can occur after infection by the parasite *Trypanosoma cruzi*[37] in Chagas' disease, where the regular occurrence of normal MMC is suppressed.

The enhanced intestinal motor response to CCK-8 after i.p. administration in postinfected rats was reproduced by oral administration of SBTI, which is a potent stimulus for the release of CCK from the upper small intestine of the rat.[38] The similar enhancement of the intestinal motor response after SBTI or CCK-8 indicates that the intraperitoneal administration of CCK-8 is relevant for the study of physiological actions of CCK in our model. However, at least in humans, endogenous CCK released after a meal includes several molecular forms, CCK-8 being released in small amounts and CCK-58 or CCK-39 appearing as the major molecular form.[39] Moreover, the biological activity of these forms are different, CCK-58 being five times more potent than CCK-8 in releasing amylase from pancreatic acini.[40] On the other hand, the doses of 10 μg kg^{-1} of CCK and of 10 mg of SBTI orally probably induce an increase in plasma CCK in the range of that induced by a balanced meal. Plasma CCK concentrations of around 3, 9 and 6 pmol L^{-1} were observed after 4 μg kg^{-1} CCK-8 i.p., 10 mg SBTI orally and a balanced meal, respectively.[41–43] We have shown in a previous study[21] that the MMC disruption induced by a meal known to be a

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potent releaser of CCK was not different in control and postinfected rats, and this can be considered to contrast with the enhanced response to CCK observed in the present study. However, CCK is known to be involved at central and peripheral levels in the postprandial disruption of MMC,[13,12] but other numerous neuronal and hormonal mediators have also been found to be involved in this disruption.

The CCK-8-induced inhibition of colonic contraction observed in our study is consistent with previous data obtained in rats[44] as well as in humans.[45] No change in the inhibitory colonic motor response to CCK was observed in postinfected rats, in comparison with controls. This agrees with the noxious hypersensitivity found localized to the jejunum in postinfected rats.[30] This jejunal localisation indicates that the alterations in the motor response to CCK depends upon the neuroimmune alterations induced by the parasite, with no change in mast cell numbers being observed at colonic level.[30,21]

Mast cell stabilization or depletion did not modify the MMC disruption induced by CCK-8 in both control and postinfected rats. This is not in agreement with results from Juanola *et al.*[16] showing that CCK disrupts MMC through a mechanism involving mast cell degranulation. However, this mechanism has been shown for endogenous CCK, and, as there are different forms of CCK released[39] with different biological activities,[40] it cannot be extrapolated to the effects of exogenously administered CCK-8. Moreover, there is no direct evidence for mast cell degranulating properties of CCK, and the presence of CCK receptor on mast cell has not been investigated. Until now, all digestive alterations described in *N. brasiliensis* postinfected rats, such as the increased sensitivity to distension[30] and the increased intestinal motor response to acetylcholine or neurokinin A,[21] have been found to involve the mast cell hyperplasia. The enhanced response to CCK can indeed be attributed to neuronal alterations in the intestinal wall.

A mediation by acetylcholine of the intestinal contractile action of CCK is well established[46] and CCK is known to be a potent releaser of acetylcholine in the gastrointestinal tract.[47] Moreover, a proportion of enteric neurones immunoreactive for choline acetyltransferase also show CCK immunoreactivity.[48] Intestinal inflammation induced by *T. spiralis* in the rat has been shown to induce in smooth muscle/myenteric plexus preparations an increase in uptake of the acetylcholine precursor, choline, associated with defects in acetylcholine storage and exocytosis[49]. These alterations have been considered as long-lasting and can presumably persist after the inflammation has resolved. They may be involved in the increased duration of the effect of CCK in postinfected rats.

Vagotomy abolished the enhanced jejunal motor response to CCK in postinfected rats. This is consistent with the activation of vagal afferents by CCK which is well-documented, and with several actions of CCK administered i.p., such as decrease in food intake[50,51] or inhibition of gastric emptying[52] which depend upon the integrity of the vagus. However, vagotomy did not modify the jejunal response to CCK in control rats but affected the response in postinfected rats which have jejunal mastocytosis. This observation agrees with the contact between vagal afferent nerve fibres and mast cells found in the rat small intestine.[53] More generally, a role of the vagus nerve in the action of CCK in postinfected rats supports the role attributed to this nerve as a link between the immune and the central nervous system.[54] Furthermore, the attenuation rather than elimination of the action of CCK in rats with subdiaphragmatic vagotomy suggests that the residual effect of CCK in vagotomized rats or rats treated with atropine is not due to actions carried by vagal fibres. Thus, we may hypothesize a role of the enteric nerve fibres as nerve remodelling has been described at this level.[22]

Our results point out the participation of CCK-A and CCK-B receptors in postinfected rats but only CCK-A receptors in controls. We found that both CCK-A and CCK-B receptor antagonists abolished the enhanced jejunal motor response to CCK in postinfected rats. These compounds had very few effects in control rats as only the CCK-A receptor antagonist devazepide significantly reduced the CCK response at the highest dose of 500 $\mu g\ kg^{-1}$. In postinfected rats, the efficacy of both devazepide and L-365260 concurs with the presence of CCK-A and CCK-B receptors on smooth muscle cells[55] and myenteric neurons.[56] The efficacy of the antagonists in postinfected rats only indicates that the infection induced profound and long-lasting changes in the population of CCK receptors. However, there is no evidence for a role of intestinal inflammation in these changes. An autoradiographic study indicates no change in CCK receptors in ulcerative colitis or in Crohn's disease.[57] A recent study in man[58] has evidenced an altered CCK receptor structure in a subgroup of patients with gallstones. As both CCK-A and CCK-B receptors have been identified in the vagus nerve[59] and as vagotomy abolishes the enhanced response to CCK in postinfected rats, it can be postulated that the changes in CCK receptors occur at the level of the vagus nerve. Devazepide or L-365260 lacked the ability to decrease the response to CCK-8 in vagotomized postinfected rats, confirming that CCK-A and CCK-B receptors are predominantly localized on vagal afferent fibres.

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In conclusion, the postinflammatory neuroimmune alterations of the jejunum induced by *N. brasiliensis* infection lead to a markedly increased jejunal motility response to CCK. This enhanced reactivity involves a cholinergic pathway, a vagal mediation and changes in CCK receptors, but does not depend upon the degranulation of overabundant intestinal mast cells.

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