Role of mast cells and pro-inflammatory mediators on the intestinal secretion induced by cholera toxin

Marcos F.G. Rochab,*, José E.P. Aguiara, José J.C. Sidrimc, Raimundo B. Costa b, Regina F.G. Feitosaa, Ronaldo A. Ribeiroa, Aldo A.M. Limaa

a Department of Physiology and Pharmacology, Clinical Research Unit-HUWC, Institute of Biomedicine, Faculty of Medicine, Federal University of Ceará, Fortaleza, CE, Brazil
b Faculty of Veterinary, State University of Ceará, Fortaleza, CE, Brazil
c Department of Pathology and Legal Medicine, Faculty of Medicine, Federal University of Ceará, Rua Jabaquara 344 Castelao, CEP 60, Fortaleza, Ceará 861-200, Brazil

Received 19 February 2003; accepted 16 May 2003

Abstract

Recent data suggest that diarrhea caused by Vibrio cholerae involves a pro-inflammatory mediators release, such as cytokines, prostaglandin and nitric oxide. The aim of this study was to investigate the role of mast cells and their mediators in the intestinal secretion induced by cholera toxin. We examined the dose responses, time course and role of mast cells and pro-inflammatory mediators in cholera toxin intestinal secretory response, in vivo. Cholera toxin caused a dose-dependent secretion, in ligated small intestine loops, at 18 h. Rats treated with 48/80 compound or ketotifen had a significant decrease in the intestinal secretory response. Cholera toxin secretion was significantly reduced by an unspecific histamine-serotonin receptor antagonist, histamine receptor antagonist, phospholipase A 2 and cyclooxygenase inhibitors, platelet-activating factor (PAF) receptor antagonists and TNF-α synthesis blockers. On the other hand, pretreatment with a specific serotonin receptor antagonist and lipoxygenase inhibitors failed to block this effect. Analysis of the intestinal fluid from rats injected with cholera toxin, revealed that cholera toxin induces the release of IL-1β and TNF-α into fluid. The data suggest that, at least in part, mast cells are involved in cholera toxin-induced secretion, as well as point to the importance of histamine, prostaglandins, PAF, IL-1β and TNF-α in this process.

Keywords: Cholera toxin; Intestinal secretion; Mast cells and pro-inflammatory mediators

1. Introduction

Vibrio cholerae produces intestinal secretion of water and electrolytes primarily through its enterotoxin (cholera toxin; molecular weight 84 kDa). The precise mechanism by which cholera toxin affects fluid and electrolyte secretion remains to be clarified. Many authors have suggested that prostaglandins, platelet-activating factor (PAF), cytokines, nitric oxide, neurotransmitters and the enteric nervous system may be involved (Triadafilopoulos et al., 1989; Peterson and Ochoa, 1989; Lima, 1994; Castagliulo et al., 1994; Jodal and Lundgren, 1995; Sears and Kaper, 1996; Thielman et al., 1997; Janoff et al., 1997; Soriani et al., 2002).

Some reports suggest that cholera toxin, as well as certain V. cholerae strains, may stimulate a modest intestinal inflammatory response (Silva et al., 1996; Sears and Kaper, 1996; Saha et al., 2000; Qadri et al., 2002). Saha et al. (2000) showed the presence of faecal leukocytes and erythrocytes in stools of patients of cholera caused by V. cholerae O1 and O139, which indicates some inflammatory changes in the gut mucosa. In addition, it was
demonstrated that the levels of nonspecific mediators of the innate defense system, such as PGE₂, LTB₄ and lactoferrin, as well as myeloperoxidase, were elevated at the acute stage of the disease in stools obtained from adults and children infected with *V. cholerae* O1 and O139 (Qadri et al., 2002). These authors are unanimous in that further study is required to elucidate the pro-inflammatory mechanisms involved in the underlying process.

The mechanism by which cholera toxin might stimulate the pro-inflammatory mediators in the submucosa is unknown. Potentially, diffusion of certain mediators from the epithelial cells may stimulate the submucosal cells, which, in turn, amplifies the process. Soriani et al. (2002) reported that, when epithelial cells first encounter cholera toxin produced by *V. cholerae* they secrete, not only chloride ions responsible for causing diarrhea, but also a number of cytokines that may contribute to the toxin’s potent immunomodulatory properties.

The view of the epithelial function being controlled solely by the endocrine and nervous systems has been expanded by the realization that immune cells, such as macrophages, mast cells and neutrophils, also have an important part to play in the control of intestinal physiology (McKay and Perdue, 1993; Perdue and McKay, 1994; Rocha et al., 1998; Rocha et al., 2000). Therefore, the aim of this study was to investigate the role of mast cells and their mediators in the intestinal secretion induced by cholera toxin.

2. Materials and methods

2.1. Ligated small intestinal segments

Wistar rats (150–200 g) of both sexes were fasted overnight and were provided water ad libitum. This experimental protocol was made as previously described by De and Chatterje (1953). The rats were anesthetized with an initial intramuscular dose of ketamine (70 mg/kg) and xylazine (5 mg/kg). Ligated small intestinal segments of approximately 5 cm were made with double ties of black-monofilament nylon between the segments. Phosphate buffered saline (PBS) alone or PBS plus purified cholera toxin (0.1, 0.3 and 1.0 μg/ml), in a volume of 0.5 ml, were injected into each segment. These ligated segments were then returned to the abdominal cavity without interruption of the blood supply. The rats were sacrificed after 6, 10, 14 or 18 h with thiopental sodium (25 mg/kg), and the volume (μl) to length (cm) ratio for each loop was obtained. Pharmacological studies was used prior to cholera toxin (1.0 μg/ml) injection and intestinal fluid was recovered 18 h later. All drugs were used by subcutaneous route 30 min prior cholera toxin injection into intestinal lumen, except for meloxicam, celecoxib and MK 886 (PO; 60 min).

2.2. Depletion of mast cell population

The method used has been already described (Di Rosa et al., 1971; Rocha et al., 1997; Souza et al., 1997). The rats were pretreated with 48/80 compound for 4 days (0.6 mg/kg, twice a day for 3 days and 1.2 mg/kg, IP, twice on the fourth day). On the fifth day, the depletion of the mast cell population, in a selected group of animals, was assessed by conventional light microscopy after staining the mast cells, in the peritoneal exudate, with toluidine blue. The counts obtained were compared with the number of the same cells obtained from a group of non-treated rats. Cholera toxin (1.0 μg/ml) was then injected into intestinal lumen from the remain rats (48/80 compound-treated) and the cholera toxin-induced secretion was evaluated 18 h later.

2.3. TNF-α and IL-1β assay

The concentrations of IL-1β and TNF-α in the fluid from the intestinal segment treated with cholera toxin (1.0 μg/ml) or PBS were determined by an specific ELISA (Enzyme-Linked Immune Sorbent Assay rat IL-1β or rat TNF-α Biosource International, Camarillo, CA, USA), as previously described by Rocha et al. (2000). Briefly, 50 and 100 μl of standard diluent from the TNF-α and IL-1β kits, respectively, were distributed in each well, containing antibody to TNF-α or IL-1β. Intestinal fluid sample or standards of 50–100 μl with their respective dilutions were then placed in the wells, and the second specific antibody against anti-TNF-α (immediately) or anti-IL-1β 3 h after the incubation at 37 ºC was added. Plates were incubated at room temperature (25 ºC), for an hour and a half (TNF-α or for an hour (IL-1β) The wells were then washed and 100 μl of streptavidin-HRP was added to each well. The plates were covered and left for 45 min (TNF-α) or 30 min (IL-1β) at room temperature (25 ºC), then emptied and washed again. Finally, 100 μl of stabilized chromogen was added to each well, the plates were incubated for 30 min at 25 ºC in a dark place, 100 μl of the stop solution was added, and absorbance readings were taken at 450 nm. TNF-α and IL-1β were expressed as pg/ml based on extrapolations from standard curves.

2.4. Cholera toxin

Cholera toxin was purchased from List Biological Laboratories (Campbell, CA, USA). Purity: The preparation migrates as a single major band in disc gel electrophoresis (nondenaturing conditions). Activity: when examined in a ADP-ribosylation assay, the specific enzymic activity of this lot is at least as high as that of a standard *cholera* toxin preparation. Cholera toxin purity and activity were guaranteed by the certificate of cholera toxin analysis from List Biological Laboratories. Cholera toxin was diluted in sterile PBS.
2.5. Drugs preparations

Quinacrine (20 mg/kg, SC; diluted in 5% NaHCO₃ solution), indomethacin (2 mg/kg, SC; diluted in 5% NaHCO₃ solution), nordihydroguaiaretic acid (NDGA; 60 mg/kg; SC; diluted in ethanol), pentoxifylline (50 mg/kg, SC), ketotifen (10 mg/kg, SC), 48/80 compound (0.6 mg/kg, twice a day for 3 days and 1.2 mg/kg, IP, twice on the fourth day), cyproheptadine (2 mg/kg, SC), diphenhydramine (9 mg/kg, SC) and metergoline (5 mg/kg, SC) were obtained from Sigma Chemical Company (St Louis, MO, USA). MK 886 (5 mg/kg, PO; diluted in methylcellulose) was purchased from Merck Sharp and Dohme (Rahway, NJ, USA). Dexamethasone (0.5 mg/kg, SC) was purchased from Merck Sharp and Dohme (São Paulo, SP, Brazil). Meloxicam and celecoxib (20 mg/kg, PO; diluted in distilled water) were purchased from Boehringer Ingelheim (São Paulo, SP, Brazil) and Searle & Pfizer (São Paulo, SP, Brazil), respectively. Thalidomide (50 mg/kg, SC) was obtained from ICN Biomedical, Inc. (Aurora, OH, USA). BN 52021 (20 mg/kg, SC) and WEB 2086 (20 mg/kg, SC) were obtained from the Institute Pasteur, Paris, France. The drugs whose diluent was not specified above were diluted in PBS. The doses used for each drug were based on our previous reports (Rocha et al., 1997; Souza et al., 1997; Feitosa et al., 2002).

2.6. Statistical analysis

The statistical significance of the differences between various groups was assessed by the use of analysis of variance (Bonferroni method). The results are presented as the mean ± standard error of the mean (SEM). A p value ≤ 0.05 was considered to indicate statistical significance.

3. Results

Cholera toxin (0.1, 0.3 and 1.0 μg/ml) caused a potent dose-dependent secretion in ligated small intestine loops, at 18 h (90.1 ± 26.0; 127.6 ± 18.9 and 160.6 ± 14.1 μl/cm, respectively; vs. PBS: 17.2 ± 8.6 μl/cm; Fig. 1A).

Fig. 1B shows the time-course of cholera toxin-induced intestinal secretion in rat ligated small intestinal segments. The cholera toxin (1.0 μg/ml) secretion was investigated at 6–18 h post-inoculation. This effect was significant from 10 h (139.4 ± 29.0 μl/cm) and steadily increased over 14 h (151.2 ± 20.0 μl/cm) and 18 h (187.0 ± 25.0 μl/cm).

To further evaluate if cholera toxin-induced secretion is dependent on mast cells, we treated a group of rats with 48/80 compound or with ketotifen. As shown in Fig. 2, it was observed that cholera toxin-induced secretion was significantly reduced in rats treated with 48/80 compound, which induces degranulation and decreases the mast cell population (reduction: 45%); as well as in rats treated with a mast cell stabilizer, ketotifen (reduction: 52%).

In order to examine if pro-inflammatory mediators might be involved in cholera toxin intestinal secretion, we studied the effects of many pharmacological agents in vivo. As shown in Table 1, cholera toxin intestinal secretion was partially reduced (p < 0.05) by an unspecific histamine-serotonin receptor antagonist (cyproheptadine; reduction: 48%) and by histamine receptor antagonist (diphenhydramine; reduction: 45%). However, metergoline, a serotonin receptor antagonist failed (p > 0.05) to inhibit this effect. Phospholipase A₂ inhibitors significantly blocked cholera toxin-induced intestinal secretion (dexamethasone and quinacrine; reduction: 43 and 35%, respectively). Indomethacin (unspecific cyclooxygenase inhibitor), meloxicam (cyclooxygenase-2 inhibitor) and celecoxib (cyclooxygenase-2 inhibitor) also reduced (p < 0.05) cholera toxin secretion, in vivo (reduction: 82, 71 and 60%, respectively). On the other hand, the pretreatment with NDGA (preferential lipooxygenase inhibitor) or MK 886 (specific lipooxygenase...
inhibitor) failed to significantly block this effect. Finally, PAF receptor antagonists (BN 52021 and WEB 2086) and TNF-α synthesis blockers (pentoxifylline and thalidomide) also reduced (p < 0.05) cholera toxin-induced secretion in ligated rat small intestinal segments (reduction: 32, 32, 62 and 44%, respectively; Table 1).

The analysis of the intestinal fluid from rats injected with cholera toxin (1.0 μg/ml), by an ELISA for rat IL-1β and TNF-α revealed that cholera toxin induces the release of these cytokines into fluid (IL-1β: 299.0 ± 20.0 pg/ml and TNF-α: 118.0 ± 23.0 pg/ml; Fig. 3).

4. Discussion

More recently, some authors have demonstrated that diarrhea due to V. cholerae O1, V. cholerae O139 and El Tor V. cholerae, involves an inflammatory response. However, they are unanimous in that further study is require to elucidate the inflammatory mechanism involved in the underlying process (Silva et al., 1996; Saha et al., 2000; Qadri et al., 2002).

We have provided evidence, in this study, that cholera toxin induces marked intestinal secretion in rats, in vivo. We measured intestinal fluid secretion in isolated ileal loop segments from rats, instead of rabbit, because we intended to use a lot of animals as well as because we know the effective doses and route of administration of many pharmacological agents in rats. This choice was not prejudicial to our investigation, because rat ligated intestinal segments shown to be an excellent model to study the intestinal secretion induced by cholera toxin.

We also noted that the injection of purified V. cholerae LPS (1.0 μg/ml) into isolated ileal loop segments from rats, in vivo, did not induce intestinal secretion (data not shown). Corroborating this data, Janoff et al. (1997) demonstrated that, in contrast to the effects of cholera toxin, purified V. cholerae LPS (1.0–100 μg/ml) elicited no appreciable fluid accumulation over 18 h when injected alone into the lumen of the rabbit ileal loops. Therefore, data from these

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Secretion (μl/cm) (mean ± SEM)</th>
<th>Inhibition (%)</th>
<th>Statistical significance</th>
<th>Pharmacological action</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>24</td>
<td>22.9 ± 8.1</td>
<td>–</td>
<td>–</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>CTx (1.0 μg/ml; IL)</td>
<td>26</td>
<td>194.8 ± 11.3</td>
<td>–</td>
<td>–</td>
<td>Cholera toxin</td>
</tr>
<tr>
<td>CTx + dexamethasone (0.5 mg/kg; SC)</td>
<td>24</td>
<td>111.8 ± 18.0</td>
<td>43</td>
<td>p &lt; 0.05</td>
<td>Phospholipase A2 and cytokines synthesis inhibitor</td>
</tr>
<tr>
<td>CTx + quinacrine (20 mg/kg; SC)</td>
<td>21</td>
<td>127.0 ± 24.0</td>
<td>35</td>
<td>p &lt; 0.05</td>
<td>Phospholipase A2 inhibitor</td>
</tr>
<tr>
<td>CTx + indomethacin (2 mg/kg; SC)</td>
<td>18</td>
<td>34.7 ± 12.3</td>
<td>82</td>
<td>p &lt; 0.01</td>
<td>Unspecific cyclooxygenase inhibitor</td>
</tr>
<tr>
<td>CTx + meloxicam (20 mg/kg; PO)</td>
<td>12</td>
<td>37.2 ± 19.7</td>
<td>71</td>
<td>p &lt; 0.01</td>
<td>Cyclooxygenase-2 inhibitor</td>
</tr>
<tr>
<td>CTx + celecoxib (20 mg/kg; PO)</td>
<td>9</td>
<td>78.0 ± 26.3</td>
<td>60</td>
<td>p &lt; 0.05</td>
<td>Cyclooxygenase-2 inhibitor</td>
</tr>
<tr>
<td>CTx + NDGA (60 mg/kg; SC)</td>
<td>15</td>
<td>189.1 ± 28.1</td>
<td>NS</td>
<td>NS</td>
<td>Preferential lipoxygenase inhibitor</td>
</tr>
<tr>
<td>CTx + MK 886 (5 mg/kg; PO)</td>
<td>15</td>
<td>163.0 ± 34.9</td>
<td>NS</td>
<td>NS</td>
<td>Specific lipoxygenase inhibitor</td>
</tr>
<tr>
<td>CTx + BN 52021 (20 mg/kg; SC)</td>
<td>21</td>
<td>133.0 ± 19.9</td>
<td>32</td>
<td>p &lt; 0.05</td>
<td>PAF receptor antagonist</td>
</tr>
<tr>
<td>CTx + WEB 2086 (20 mg/kg; SC)</td>
<td>14</td>
<td>133.4 ± 23.6</td>
<td>32</td>
<td>p &lt; 0.05</td>
<td>PAF receptor antagonist</td>
</tr>
<tr>
<td>CTx + pentoxifylline (50 mg/kg; SC)</td>
<td>15</td>
<td>74.0 ± 17.4</td>
<td>62</td>
<td>p &lt; 0.05</td>
<td>TNF-α synthesis inhibitor</td>
</tr>
<tr>
<td>CTx + thalidomide (50 mg/kg; SC)</td>
<td>15</td>
<td>109.5 ± 19.1</td>
<td>44</td>
<td>p &lt; 0.05</td>
<td>TNF-α synthesis inhibitor</td>
</tr>
<tr>
<td>CTx + cyproheptadine (2 mg/kg; SC)</td>
<td>9</td>
<td>102.2 ± 34.3</td>
<td>48</td>
<td>p &lt; 0.05</td>
<td>Histamine and serotonin receptor antagonist</td>
</tr>
<tr>
<td>CTx + diphenhydramine (9 mg/kg; SC)</td>
<td>9</td>
<td>108.0 ± 24.3</td>
<td>45</td>
<td>p &lt; 0.05</td>
<td>Histamine receptor antagonist</td>
</tr>
<tr>
<td>CTx + metergoline (5 mg/kg; SC)</td>
<td>9</td>
<td>197.5 ± 27.1</td>
<td>NS</td>
<td>NS</td>
<td>Serotonin receptor antagonist</td>
</tr>
</tbody>
</table>

n: number of intestinal segments; CTx: cholera toxin; NS: not significative.
stimulated with CTx (1.0 μg/ml) for the levels of TNF-α indicated the release of TNF-α mean indicates the number of samples measured in duplicate. *p < 0.05 for the levels of TNF-α and IL-1β in the intestinal segments stimulated with CTx (1.0 μg/ml) vs. PBS (control).

![Graph](image)

Fig. 3. Detection of TNF-α and IL-1β into the intestinal fluid from segment treated with cholera toxin. Cholera toxin (CTx; 1.0 μg/ml) induced the release of TNF-α and IL-1β. The data are plotted as mean ± SEM and were analyzed by ANOVA (Bonferroni method). n indicates the number of samples measured in duplicate. *p < 0.05 for the levels of TNF-α and IL-1β in the intestinal segments stimulated with CTx (1.0 μg/ml) vs. PBS (control).

researches indirectly suggests that lipopolysaccharide is not involved in the intestinal secretion induced by cholera toxin.

Mast cells are immunocompetent cells that can synthesize and secrete an array of mediators, that could affect the epithelium directly or indirectly (McKay and Perdue, 1993; Perdue and McKay, 1994; Rocha et al., 1998, 2000).

We observed that the treatment of rats with 48/80 compound, which causes degranulation and decreases the mast cells population (Di Rosa et al., 1971; Rocha et al., 1997; Souza et al., 1997) as well as with a mast cell stabilizer, ketotifen (Grant et al., 1990; Feitosa et al., 2002), significantly reduced cholera toxin-induced secretion in ligated rat small intestinal segments. We have evidenced, as observed in this investigation, that the treatment of rats with systemic 48/80 compound induces a potent depletion (94–96%) in the mast cell population (Rocha et al., 1997; Souza et al., 1997). Therefore, these experiments suggest that mast cells, at least in part, appear to be involved in the secretory activity of cholera toxin.

Certain mast cell-derived mediators, such as prostaglandins (Calderaro et al., 1991), leukotrienes (Smith, 1992), PAF (Hanglow et al., 1989), histamine (Hardcastle and Hardcastle, 1987), serotonin (Beubler et al., 1986), IL-1β (Chiossone et al., 1990) and TNF-α (Kandil et al., 1992), may cause intestinal secretion. Therefore, a specific pharmacological screening was made in an attempt to investigate if one or more of these pro-inflammatory mediators could be involved in cholera toxin-induced secretion, in vivo.

The ability of quinacrine, a phospholipase A2 inhibitor, to partially reduced cholera toxin-induced secretion suggested that arachidonic acid metabolites are probably involved in this effect. This evidence was confirmed by the inhibition produced by dexamethasone, which alters the mRNA transcription for several cytokines and blocks phospholipase A2 activity. In addition, cyclooxygenase inhibitors (indomethacin, meloxicam and celecoxib) and PAF receptor antagonists (BN 52021 and WEB 2086) also reduced cholera toxin-induced secretion, in vivo. On the other hand, preferential and specific lipoxygenase inhibitors, NDGA and MK 886, respectively, had no significant effect on this secretory activity. We have demonstrated that, both NDGA and MK 886, taken in the same route of administration and dosage, are able to block the activity of leukotrienes in rats (Rocha et al., 1997; Souza et al., 1997; Feitosa et al., 2002). Therefore, these results suggest that prostaglandins and PAF, but not leukotrienes, could be involved in the cholera toxin-induced secretion.

In agreement with these results it has been reported that cholera toxin-induced prostaglandin, platelet-activating factor and cAMP formation are interrelated (Peterson and Ochoa, 1989; Guerrant et al., 1994; Sears and Kaper, 1996; Thielman et al., 1997). It was also evidenced that injection of purified cholera toxin into rabbit ileal loops causes a significant increase in fluid secretion and intestinal permeability to mannitol; as well as a release of PGE₂ into lumen, but it had no effect on LTB₄ release (Triadafilopoulos et al., 1989).

We also investigated the involvement of histamine and serotonin in cholera toxin-induced intestinal secretion, by the treatment of rats with an unspecific histamine/serotonin receptor antagonist (cyproheptadine), histamine receptor antagonist (diphenhydramine) and serotonin receptor antagonist (metergoline). Data from these experimental protocols showed that cyproheptadine and diphenhydramine, but not metergoline, decrease this cholera toxin activity. Therefore, this evidence suggests that histamine, but not serotonin, is involved in the intestinal secretion induced by cholera toxin.

Corroborating the data above, it has been reported that histamine contributes to diarrhea associated with systemic anaphylaxis, food allergy, systemic mastocytosis and rejection of helminth parasites (Ciancio and Chang, 1992). It was also observed that the addition of histamine to the serosal side of human colonic cell line monolayers (T₈₄ cells) led to transient chloride secretion, which could be inhibited by diphenhydramine (Barret, 1992). In addition, histamine has been reported to indirectly stimulate chloride secretion in intestinal segments from guinea pig and rat, via stimulation of arachidonic acid metabolism and enteric nerve stimulation (Cook et al., 1984; Hardcastle and Hardcastle, 1987).

In an attempt to investigate the possible role of IL-1β and/or TNF-α in cholera toxin-induced secretion, we used a pharmacological screening with pentoxifylline and thalidomide; an enzyme-linked immunosorbent assay for rat IL-1β and TNF-α was also used. Pretreating of rats with pentoxifylline and thalidomide resulted in partial inhibition of the cholera toxin intestinal effect. In addition, high levels of IL-1β and TNF-α were detected in the intestinal fluid recovered from rats injected with cholera toxin. Consequently, these data suggest that IL-1β and TNF-α are
probably involved in cholera toxin-induced secretion in rat ligated small intestinal segments.

In agreement with these data, more recently, Viana et al. (2002) demonstrated that cholera toxin exhibits a significant pro-inflammatory activity by inducing paw edema in rats. TNF-α and IL-1β may be involved in this edematogenic effect, since pentoxifylline and thalidomide were able to partially block this cholera toxin activity. Furthermore, it has been shown that TNF-α (Rodriguez et al., 1998; Yan et al., 1999) and IL-1β (Cong et al., 2001) are involved in some cholera toxin biological activity.

In conclusion, data from the present investigation suggest that mast cells, at least in part, are involved in cholera toxin-induced secretion, as well as point to the importance of histamine, prostaglandins, PAF, IL-1β and TNF-α in this process.

Acknowledgements

This work was supported in part by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) and Howard Hughes Medical Institute (Grant: 55000645).

References


