Effects of Cyclophosphamide on the Kaolin Consumption (Pica Behavior) in Five Strains of Adult Male Rats

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ABSTRACT. It is known that pica, the consumption of non-nutritive substances such as kaolin, can be induced by administration of toxins or emetic agents in rats. In the present study, we examined the effects of intraperitoneal (i.p.) administration of cyclophosphamide on pica behavior and on the concentration of 5-hydroxyindoleacetic acids (5HIAA) in cerebrospinal fluid (CSF) in the following five strains of adult male rats: Sprague Dawley (SD), Wistar, Fischer 344 (F344), Wistar-Imamichi (WI) and Long Evans (LE). Cyclophosphamide (25 mg or 50 mg/kg) was injected (i.p.) into the rats and kaolin and food intake were measured at 24 hr after injection. The animals were anesthetized with urethane (1 g/kg) at 3 hr after injection of cyclophosphamide, and CSF was collected from the cisterna magna. WI and LE rats clearly showed pica behavior as compared with the other strains. In LE rats, the concentration of 5HIAA in CSF also increased in a dose-dependent manner of cyclophosphamide. The pretreatment with ondansetron (5-HT₃ antagonist) restored both changes (kaolin consumption and 5HIAA levels) induced by cyclophosphamide. These results suggest that the LE rat is sensitive to cyclophosphamide, that pica induced by cyclophosphamide mimics many aspects of emesis including the serotonergic response in the central nervous system and that use of the pica model would be a practical method for evaluating the effects of antiemetic drugs in addition to the mechanism of emesis.

KEY WORDS: cyclophosphamide, emesis, kaolin, pica, rat.

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Cancer chemotherapy with cyclophosphamide or cisplatin is usually associated with nausea or vomiting in humans [1, 2, 5, 8, 16]. These cytotoxic drugs can induce emesis in ferrets [8] and house musk shrews [14, 20], and these two animal species have been often used in experiments for emesis. However, studies on the mechanisms of emesis in common laboratory animals such as rats and mice are limited due to the lack of vomiting reflex in rats and mice [8]. Although rats and mice do not vomit, they show pica behavior (the consumption of non-nutritive substances such as kaolin) in response to the various emetic stimuli [7, 12, 24, 30-32]. In rats, pica behavior can be induced by administration of cancer chemotherapy agents [11-13, 18, 22], lithium chloride [18, 32], copper sulphate [32] and by motion exposure [7, 24]. This behavior is recognized as an index of emesis, and the kaolin ingestion model can be used as a quantifiable behavioral assay of toxins [8, 15]. In previous reports, Long Evans (LE) and Wistar rats were mainly used in experiments for pica behavior [11-13, 18, 22], but the reasons why these two strains have been used in this field of study are unclear. There has been no study comparing the expression of pica induced by any emetic stimuli among several strains of rats. In order to choose the best strain of rat for future pica research, we examined the effects of intraperitoneal (i.p.) administration of cyclophosphamide on pica behavior using Sprague Dawley (SD), Wistar, Fischer 344 (F344), Wistar-Imamichi (WI) and LE adult male rats in

the present study and compared the expression of pica among the five strains of male rats. Serotonergic neurons are important for induction of emesis [1, 2, 8, 15, 16], and the concentration of 5-hydroxyindoleacetic acids (5HIAA), a metabolite of 5-HT, in cerebrospinal fluid (CSF) and the effects of 5-HT antagonist on pica behavior were also examined in adult male rats treated with cyclophosphamide.

MATERIALS AND METHODS

Animals and kaolin preparation: Adult male Wistar-Imamichi (WI) and LE rats were obtained from the Institute for Animal Reproduction (Ibaraki, Japan), and Wistar, F344 and SD rats were obtained from Charles River Laboratories Japan Inc. (Kanagawa, Japan). All animals were obtained at 7 weeks of age, were housed under controlled temperature and lighting (lights on from 0700 to 1900 hr) conditions and supplied with food (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and water *ad libitum*. All animals were housed in isolation cage made of wire mesh (150 mm x 260 mm x 180 mm) at 3 days prior to the onset of each experiment. Five to 6 rats (9-10 weeks old) of each strain were used in each experiment. The body weights of each strain before the experiment were 398.55 ± 10.32 g for WI, 419.62 ± 6.79 g for W, 385.02 ± 5.39 g for SD, 423 ± 6.45 ± 6.24 g for LE and 222.58 ± 1.95 g for F344. All procedures were approved by the animal care and use committee of Dokkyo Medical University.

Kaolin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) pellet was prepared according to the previous method [18]. Kaolin was mixed with 1% Arabic gum (Wako Pure

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Chemical Industries, Ltd., Osaka, Japan) in distilled water to form a shape that was similar to chow pellets and dried completely at room temperature.

Effects of cyclophosphamide on kaolin consumption, food consumption and body weights in the five rat strains: The kaolin was given 3 days before the cyclophosphamide treatment to allow the rats to be acclimated to the presence of kaolin in the cage [12, 13, 22]. Kaolin and food were provided in a separate compartment in the food containers. On the day of the experiment, the rats were weighed and given an intraperitoneal (i.p.) injection of either saline (2 ml/kg) or cyclophosphamide (25 and 50 mg/kg) at 9:00 hr. Cyclophosphamide (Sigma, St. Louis, MO, U.S.A.) was dissolved in sterile saline (25 mg/ml). Spilled kaolin and food were collected, and kaolin and food consumption and body weight were recorded at 24 hr after administration of cyclophosphamide. The results for body weight were expressed as the ratio of the changes at 24 hr after administration to body weight at the previous day.

Effects of cyclophosphamide on 5HIAA in CSF and on corticosterone in plasma in the five rat strains: Adult male rats were injected with urethane (1 g/kg) intraperitoneally at 3 hr after administration of either saline or cyclophosphamide (25 and 50 mg/kg), and CSF was collected from the cisterna magna using a Kopf stereotaxic instrument. Trunk blood was collected by decapitation just after sampling of CSF. Urethane anesthesia was chosen because it is widely used for neuroscience research [15, 23, 24] including CSF analysis [15, 24], and it has been reported that pentobarbital sodium decreased the concentration of monoamine in CSF [24].

It has been reported that the level of 5HIAA, a metabolite of 5-HT, reflects the activity of serotonergic neurons [15, 24]. To estimate the activity of serotonergic neurons in the brain after administration of cyclophosphamide, the concentration of 5HIAA in CSF was determined by high-performance liquid chromatography (HPLC) with electrochemical detection (ECD) [3, 25, 29]. The mobile phase (pH 3.0) consisted of 40 mM citric acid, 40 mM dibasic sodium phosphate, 0.08 mM EDTA, 7.5 mM sodium heptanesulphonate, 4% methanol, and 2% acetonitrile [23]. The retention time for 5HIAA was 12.1 min.

To investigate the adrenal response against chemical stress (administration of cyclophosphamide) in the 5 rat strains, plasma concentrations of corticosterone were determined by double-antibody radioimmunoassay using ^{125}I -labeled radioligand as described previously [9, 28]. The intra- and interassay coefficients of variation were 6.3% and 11.9% for corticosterone.

Effects of ondansetron (5-HT type 3 antagonist) on kaolin consumption, food consumption, body weights and 5HIAA levels in CSF in LE rats treated with cyclophosphamide: To investigate the effect of an anti-emetic agent (ondansetron) on pica behavior, ondansetron (4 mg/kg dissolved in saline) or saline was injected (i.p.) into rats at 30 min prior to treatment (i.p.) with cyclophosphamide (37.5 mg/kg) or saline as a control. The dose of cyclophosphamide or

ondansetron was chosen as a result of the preliminary experiment. In the preliminary experiment, 3 doses of cyclophosphamide (25 mg/kg, 37.5 mg/kg and 50 mg/kg) were tested, and the middle dose (37.5 mg/kg) was the most effective in this experiment.

In one experimental group, kaolin and food consumption and body weight in LE adult male rats were recorded at 24 hr after administration of cyclophosphamide.

In the other experiment, CSF was collected in LE rats under urethane anesthesia at 3 hr after administration of cyclophosphamide, and the concentration of 5-HIAA in CSF was determined by HPLC with ECD.

Statistical analyses: All results are expressed as the mean $\pm$ SEM. The data were analyzed using one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test; a value of $P < 0.05$ was considered significant.

RESULTS

Effects of cyclophosphamide on kaolin consumption, food consumption and body weight in five rat strains: Kaolin consumption was increased at 24 hr after administration of cyclophosphamide in all rat strains, although the increase was not significant in the Wistar strain (Fig. 1-a). On the other hand, food consumption (Fig. 1-b) and body weight (Fig. 1-c) were decreased in all rat strains as a result of cyclophosphamide treatment. In the WI, LE and SD strains, treatment with lower dose (25 mg/kg) also increased kaolin consumption, and the WI and LE rats clearly showed pica behavior as compared with the other strains (Fig. 1-a). In these two strains, kaolin consumption increased in a dose-dependent manner of cyclophosphamide.

Effects of cyclophosphamide on 5HIAA in CSF and on corticosterone in plasma in the five rat strains: The concentration of 5HIAA in CSF increased at 3 hr after administration of cyclophosphamide (50 mg/kg) in all rat strains, but the increase was not significant in the WI strain (Fig. 2-a). The plasma concentrations of corticosterone also increased in all strain rats as a result of cyclophosphamide treatment, but the increase was not significant in the SD and F344 rats (Fig. 2-b).

Effects of ondansetron (5-HT type 3 antagonist) on kaolin consumption, food consumption, body weights and 5HIAA levels in CSF in LE rats treated with cyclophosphamide: Administration of cyclophosphamide (37.5 mg/kg) increased kaolin consumption (Fig. 3-a) and decreased food consumption (Fig. 3-b) and body weights (Fig. 3-d) at 24 hr after administration in LE rats. Pretreatment with ondansetron (5-HT₃ antagonist) restored these changes (kaolin consumption, food consumption and body weights) induced by the cyclophosphamide treatment (Fig. 3-a, b, d). The concentration of 5HIAA in CSF was also increased at 3 hr after administration of cyclophosphamide, and the 5HIAA level was restored in LE rats pretreated with ondansetron (Fig. 3-c).

Fig. 1. Effects of intraperitoneal injection of cyclophosphamide (25 mg/kg or 50 mg/kg) on kaolin consumption (a), food consumption (b) and body weights (c) in adult male Wistar-Imamichi (WI), Wistar (W), Sprague Dawley (SD), Long Evans (LE) and Fischer 344 (F344) rats. All data were collected at 24 hr after injection of cyclophosphamide. Each point represents the mean $\pm$ SEM of 5 animals. Asterisks indicate $P < 0.05$ compared with the value for the saline-treated control (one-way ANOVA followed by Fisher's PLSD test).

DISCUSSION

Emesis is one of the major side effects of cancer chemotherapy [1, 2, 5, 8, 16] and is also associated with X-ray irradiation [8] and motion sickness [7, 24]. Ferrets and house

Fig. 2. Effects of intraperitoneal injection of cyclophosphamide (25 mg/kg or 50 mg/kg) on the concentration of 5HIAA in CSF (a) and of corticosterone in plasma (b) in adult male Wistar-Imamichi (WI), Wistar (W), Sprague Dawley (SD), Long Evans (LE) and Fischer 344 (F344) rats. Animals were anesthetized with urethane (1 g/kg) at 3 hr after injection of cyclophosphamide, and CSF was collected from the cisterna magna. For plasma sampling, trunk blood was collected by decapitation just after sampling of CSF. Each point represents the mean $\pm$ SEM of 5 to 6 animals. Asterisks indicate $P < 0.05$ compared with the value for the saline-treated control (one-way ANOVA followed by Fisher's PLSD test).

mus mus are the experimental animals used for emesis research because these two species show the reflex of vomiting [8]. On the other hand, common laboratory animals such as rats and mice lack the vomiting reflex, but they show pica behavior (the consumption of non-nutritive substances such as kaolin) in response to the various emetic stimuli [7, 12, 24, 30–32]. The sequences of the rat and mouse genomes have already been established [19, 21], and using a rat or mouse for any field of biomedical research has the benefit of advancing the research scheme. In the case that the pica model mimics many aspects of emesis, choosing a rat or mouse pica model would be advantageous for emesis

Fig. 3. Effects of the pretreatment (30 min prior to cyclophosphamide treatment) of ondansetron (4 mg/kg) on kaolin consumption (a), food consumption (b), 5HIAA levels in CSF (c) and body weights (d) in adult male Long Evans (LE) rats treated with cyclophosphamide (37.5 mg/kg). The kaolin and food consumption and body weights were recorded at 24 hr after administration of cyclophosphamide, and the 5HIAA in CSF was determined by HPLC with ECD at 3 hr after administration of cyclophosphamide. Each point represents the mean $\pm$ SEM of 5 to 6 animals. *: $P < 0.05$ compared with the control (saline). #: $P < 0.05$ compared with the ondansetron-treated group (one-way ANOVA followed by Fisher's PLSD test).

research, but there has been no study comparing the expression of pica among several strains of rats. In the present study, we compared the expression of pica induced by cyclophosphamide treatment among five strains (SD, Wistar, F344, WI and LE) of male rats.

Administration of cyclophosphamide increased kaolin consumption and decreased food consumption and body weights in all five rat strains, but WI and LE showed clearer behavioral response than the other three strains. These two strains (WI and LE) also showed a higher response of corticosterone secretion after administration of cyclophosphamide. The sensitivity of pica expression was concomitant with the sensitivity of adrenal response to chemical stress. A significant increase of the 5HIAA levels in CSF was observed in the LE rats treated with cyclophosphamide, but the increase was not significant in the WI rats. The base level of 5HIAA in the saline-treated WI rats was comparatively higher than in the other four strains, and the urethane anesthesia may have affected the value of 5HIAA in the WI rats. It may be necessary to examine the contents of 5-HT in the area postrema of the medulla oblongata using unanesthetized rats in a future study. The present results suggest that the LE rat is a suitable strain for pica research in terms of kaolin consumption, the sensitivity to chemical stress and the activity of serotonergic neurons.

Previous toxicological research reports have mentioned

that LE rats were more sensitive to the toxic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the liver [9] and the effects of tri-o-cresyl phosphate (inhibition of acetylcholinesterase) in the brain [4] compared with SD and F344 rats. The gene expression of hepatic cytochrome P450 1A1 induced by TCDD in LE rats is significantly increased compared with those of SD, F344, WI and Lewis rats [9]. The strain difference of the gene expression of hepatic enzymes induced by toxic or pharmacologic compounds may also account for the lack of increase in 5HIAA in the WI strain and may also account for the strain differences of pica behavior induced by cyclophosphamide.

The serotonergic neuron is important for the expression of emesis or pica induced by anticancer drugs [1, 2, 5, 8, 16]. It is proposed that anticancer drugs such as cyclophosphamide and cisplatin evoke 5-HT release from the enterochromaffin cells in the intestinal mucosa and that the released 5-HT causes depolarization of the vagal afferent nerves mediated by the 5-HT receptor (especially 5-HT₃). The vagal afferent nerve stimulates the vomiting center in the brainstem and finally induces a vomiting reflex [1, 2, 5, 8, 16]. Antiemetic drugs administered for the side effects of cancer chemotherapy are thought to act at the 5-HT receptor on the adjacent vagal afferent nerve in the human, ferret or house musk shrews [2, 5, 16]. Administration of a 5-HT₃ antagonist has also been reported to attenuate kaolin consumption

induced by anticancer drugs [13, 22] and X-ray irradiation [31] in rats, but there is no report examining the effects of 5-HT₃ antagonist on the 5-HT or 5HIAA levels in the central nervous system (CNS) in rats showing pica behavior. In the present study, we examined the effects of ondansetron on pica behavior and 5HIAA levels in CSF in rats treated with cyclophosphamide. The results showed that 5-HT₃ antagonist restored all changes (kaolin consumption, food consumption, body weights and 5HIAA levels) induced by cyclophosphamide, and suggest that the action site of ondansetron would be at 5-HT receptor on the adjacent vagal afferent nerve and that evaluating the effects of antiemetic drugs using the pica model would be a practical method for emesis research.

In summary, the LE rat is the sensitive strain in response to treatment with cyclophosphamide. Pica induced by cyclophosphamide mimics many aspects of emesis including the serotonergic response in the CNS. These results suggest that using the pica model would be a practical method for evaluating the effects of antiemetic drugs in addition to the mechanism of emesis.

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