Effects of Lipid Mediator Antagonists on Predominant Mediator-Controlled Asthmatic Reactions in Passively Sensitized Guinea Pigs

YASUHITO ARAKIDA, KEIKO OHGA, KIYOMI SUWA, MASAKI YOKOTA, KEIJI MIYATA, TOSHIMITSU YAMADA, and KAZUO HONDA

Inflammation Research Pharmacology Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., Miyukigaoka, Tsukuba-shi, Ibaraki, Japan

ABSTRACT

The role of cysteinyl leukotrienes (cys-LTs) and thromboxane A2 (TXA2) in guinea pig models of aspects of bronchial asthma was investigated. In a novel antigen (BSA)-induced asthmatic model using passively sensitized guinea pigs, pretreatment with varying doses of indomethacin controlled the ratio of followed lipid mediators, LTC4/D4/E4 and TXB2, in lungs of challenged guinea pigs. The predominant mediator in indomethacin-untreated asthma was TXA2, and complete inhibition of cyclooxygenase by i.v. injection of 5-mg/kg indomethacin-induced cys-LTs mainly mediated asthmatic response. Furthermore, a 1-mg/kg indomethacin dose induced an asthmatic state where both cys-LTs and TXA2 equally participated. Either LTD4 or TXA2 receptor antagonists given alone inhibited the asthmatic response in conditions where the corresponding mediator plays a predominant role. The combination of LTD4 and TXA2 receptor antagonists exhibited significant effects irrespective of the condition used. Under conditions where both mediators equally participate, a combination of both receptor antagonists showed additive inhibition. YM158, a newly synthesized and orally active dual antagonist for LTD4 and TXA2 receptors, showed the same antiasthmatic effect as a combined LTD4 receptor antagonist and a TXA2 receptor antagonist mixture. Therefore, broad-acting compounds such as YM158 are expected to have antiasthmatic efficacies in a broader class of asthmatic patients than single-acting drugs.

Received for publication January 28, 1999

ABBREVIATIONS: cys-LT, cysteinyl leukotrienes (LTC4, LTD4, and LTE4); TX, thromboxane; YM158, 3-[(4-tert-butylthiazol-2-yl)methoxy]-5-[3-(4-chlorobenzensulfonyl) propyl]-2-[(1H-tetrazol-5-ylmethoxy)benzanilide monosodium salt monohydrate.
the LTD₄-predominant type, the TXA₂-predominant type, and both mediator-related conditions was established using passively sensitized guinea pigs. Because the predominant lipid mediator in asthmatic guinea pigs was TXA₂, it may be possible to control the predominant mediator by a cyclooxygenase inhibitor such as indomethacin. Therefore, we tried to make the predominant mediator-controlled asthmatic models using indomethacin in passively sensitized guinea pigs. This model was then used to classify the type of asthma as in clinical practice to evaluate lipid mediator antagonists such as pranlukast, daltroban (Ogletree and Allen, 1992), and YM158, 3-[(4-tert-buty1)dia11azol-2-yl]methoxy]-5‘-[3-(4-chloro-benzencesulfonyl) propyl]-2‘-(1H-tetrazol-5'-ylmethoxy)benzannilide monosodium salt monohydrate, a newly synthesized dual antagonist for LTD₄ and TXA₂ receptors (Yokota et al., 1997; Arakida et al., 1998).

**Materials and Methods**

**Animals**

Male Hartley guinea pigs (Charles River Japan Co., Yokohama, Japan) were used, which weighed 280 to 480 g at the time of challenge. The animals were given food and water ad libitum until the day before the experiment. Food was withheld overnight to eliminate the effect of food on absorption after p.o. administration of the test compounds.

**Chemicals**

The following drugs and chemicals were used: YM158 (Arakida et al., 1998) and daltroban (4-[2-[(4-chlorophenyl) sulfonyl] amin0]ethyl benzeneacetic acid) (Ogletree and Allen, 1992) were synthesized by Yamanouchi Pharmaceutical Co., Ltd. (Tsukuba, Japan). Pranlukast (4-oxo-8-[4-(4-phenylbutylo) benzyloamino]-2-(tetrazol-5-yl)-4H-1-benzopyran hemihydrate) (Obata et al., 1992) was purified from the commercially available formulation ONON (Ono Pharmaceutical Co., Osaka, Japan). BSA (essentially globulin free), urethane, indomethacin, pyrilamine maleate salt, propranolol hydrochloride, and nordihydroguaiaretic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Evans blue was purchased from Tokyo Kasei Co. (Tokyo, Japan) and TC-5E (HPMC2910, hydroxypropylmethylcellulose) and HCO-60 was purchased from Shin-Etsu Chemical Co. (Tokyo, Japan). Pyrilamine maleate, propranolol, BSA, and Evans blue were dissolved in 0.9% saline. Indomethacin was dissolved in 0.9% saline containing a few drops of 1 N NaOH solution. The free base of YM158, pranlukast, and daltroban for oral administration was dissolved in dimethyl sulfoxide containing TC-5E (15 mg/ml) and HCO-60 (2.5 mg/ml).

**Immediate Asthmatic Responses in Passively Sensitized Guinea Pigs**

**Passive Sensitization of Guinea Pigs.** Animals were sensitized by i.v. injection of rabbit anti-BSA serum (1 ml/kg b.wt.) on the day before the experiment. To induce the same level shortage on onset time for coughing in all groups, the titer of anti-BSA serum was ×4,000 for indomethacin-pretreated animals and ×64,000 for indomethacin-nontreated animals. The antigen-induced symptom score and airway microvascular leakage were also the same level in all groups under these conditions. Animals injected with 1 ml/kg of nonsensitized rabbit serum were used as nonsensitized controls.

**Inhaled Antigen Challenge in Aerosolized Form.** Antigen-induced immediate asthmatic response was examined in passively sensitized guinea pigs. The animals were pretreated with pyrilamine (2 mg/kg i.v.) and propranolol (0.3 mg/kg i.v.) 5 min before the antigen challenge. The sensitized animals were placed in a transparent cylindrical box and challenged by inhalation of an antigen aerosol (1% BSA solution in 0.9% saline) using a glass nebulizer (Kinoshita, Tokyo, Japan) for 2 min. To control the balance of cys-LTs and TXA₂, animals were i.v. pretreated with 0, 1, or 5 mg/kg of indomethacin 20 min before the antigen challenge. The test compounds were p.o. administered 1 h before the antigen challenge.

**Determination of LTC₄/D₄/E₄ and TXB₂ Levels in Isolated Lungs.** Animals were sacrificed by exsanguination 15 min after antigen challenge. The chest cavity was opened to allow perfusion with buffer A (0.9% saline containing 10 μM indomethacin and 10 μM nordihydroguaiaretic acid) from the pulmonary artery to the left atrium. The lung parenchyma was removed and homogenized in 5 ml of buffer A. The homogenized lung tissue was added to EtOH, stored at 4°C for 30 min, and then centrifuged at 2,000g, 4°C for 15 min. The supernatant fraction was retained and dried under reduced pressure. The residue was dissolved in 5 ml of H₂O and adjusted to pH 3 to 4 by addition of a 1 M citric acid solution.

This sample was applied to an octadecysil column (SEP-PAK C18; Waters Associates, Milford, MA). To obtain the TXB₂ fraction, a stable metabolite of TXA₂, the sample applied cartridge was washed with 15% EtOH solution and petroleum ether, and then lipophilic components were eluted with 4.5 ml of ethyl acetate. These eluted fractions were dried by rotary evaporation under reduced pressure, and then these samples were dissolved in assay buffer for radioimmunoassay systems. The recovery rate in each experiment was measured by adding the radioactive lipid’s standard to samples isolated from normal guinea pigs, and the content values were corrected using this recovery rate. LTC₄/D₄/E₄ and TXB₂ levels in isolated guinea pig lung tissue extracts were then measured using a radioimmunoassay kit (Amersham International, Buckinghamshire, England).

**Observation of Onset Time for Asthmatic Response and Symptoms.** Animals were observed for 10 min, and the interval between antigen inhalation and appearance of coughing was recorded as the onset time for asthmatic response. Values for animals that did not cough within 10 min after the antigen challenge were recorded as 600 s. Symptoms observed within 30 min after the start of antigen aerosolization were recorded according to the following scale. The most severe symptom observed in each animal during the 30-min observation period was used as the symptom score for animal. Scoring was as follows: 1 = restlessness/tachypnea; 2 = coughing; 3 = convulsions; 4 = collapse; and 5 = death. In the succeeding experiments, animals that were treated with normal rabbit serum and then exposed to antigen were defined as nonsensitized animals.

**Antigen-Induced Airway Microvascular Leakage.** Five minutes before antigen challenge, 1 ml of 0.5% Evans blue solution in 0.9% saline was injected into the animals. Thirty minutes after the challenge, the animals were anesthetized with urethane (1.2 g/kg i.p.) and sacrificed by exsanguination. The chest cavity was opened and blood containing Evans blue dye was washed out by perfusion with 0.9% saline from the pulmonary artery to the left atrium. The airways and lungs were removed and cleared of extraneous connective tissues. The airways were cleared of parenchyma and then wet weight of the trachea and bronchi was measured. Evans blue dye was extracted from the airways with 3 ml of formamide by standing at 4°C for more than 24 h. The amount of Evans blue dye was determined with a spectrophotometer (UV-160A; Shimazu, Kyoto, Japan) at 620 nm and expressed as mg/mg tissue wet weight. Baseline values were determined in nonsensitized guinea pigs.

**Statistical Analysis.** Experimental results are shown as the means ± S.E. For multiple comparison between the control group and treated groups in the experiment to determine onset time for asthmatic response, statistical significance was determined using Dunnett’s multiple range test. For the experiment to determine the dye leakage, Student’s t test was used to compare the nonsensitized group with the control group, and Dunnett’s multiple range test was used for multiple comparison of the treated groups with the control group. For the symptom score, the Wilcoxon rank sum test was used to compare the control group with the nonsensitized group, and
Steel's test was used for multiple comparison of the treated groups with the control group. p values < .05 were defined as significant.

**Results**

**Lipid Mediator Content in Antigen-Challenged Guinea Pig Lung**

The predominant lipid mediator, LTC₄/D₄/E₄ as cysteinyl-LTs and TXB₂ (a stable metabolite that reflects TXA₂ levels), was measured by radioimmunoassay (Table 1). In guinea pigs not treated with indomethacin, the content of TXB₂ before antigen challenge was about 12 times higher than that of LTC₄/D₄/E₄ (0.82 ± 0.12 ng/g tissue of LTC₄/D₄/E₄ and 10.10 ± 1.08 ng/g tissue of TXB₂). After antigen challenge, an increase in both parameters was observed, and the ratio was almost the same with 8.8 for LTC₄/D₄/E₄ and 7.1 for TXB₂ contents, respectively.

In guinea pigs treated with 5 mg/kg of indomethacin, the content of TXB₂ (3.38 ± 0.46 ng/g tissue) before antigen challenge was slightly higher (2.94 times) than that of LTC₄/D₄/E₄ (1.15 ± 0.40 ng/g tissue). Furthermore, the antigen-induced increase in the ratio of LTC₄/D₄/E₄ (29.5) was about 20 times higher than that of TXB₂ (1.5) when complete inhibition of cyclooxygenase due to sufficient indomethacin occurred. Therefore, the content of LTC₄/D₄/E₄ (33.89 ± 6.75 ng/g tissue) was about 7 times higher than that of TXB₂ (4.91 ± 0.93 ng/g tissue).

In guinea pigs treated with 1 mg/kg of indomethacin, the content of TXB₂ before antigen challenge was 4.4 times higher than that of LTC₄/D₄/E₄. The antigen-induced increase in the ratio of LTC₄/D₄/E₄ and TXB₂ was almost the same with 4.7 for LTC₄/D₄/E₄ and 3.9 for TXB₂ contents. Therefore, the content of TXB₂ (16.37 ± 3.31 ng/g tissue) after antigen challenge was 3.6 times higher than that of LTC₄/D₄/E₄ (4.50 ± 0.82 ng/g tissue).

**Effects of Oral Pranlukast and Daltroban on Antigen-Induced Immediate Asthmatic Responses**

**Onset Time for Asthmatic Response and Symptoms.** Although almost no coughing was observed in guinea pigs in the nonsensitized groups, all of the guinea pigs in the sensitized control groups in each experiment coughed, and the mean values for the onset times for asthmatic response were between 196 and 222 s. The onset time for asthmatic response of sensitized guinea pig became significantly shorter when compared with nonsensitized guinea pigs in all conditions used (Fig. 1). Asthmatic symptom scores were between 2.6 and 3.6 in the control groups, and were significantly greater than those of the nonsensitized groups (1.3–1.5) (Fig. 2). In groups not treated with indomethacin, administration of daltroban (10 mg/kg), a combination of pranlukast (30 mg/kg) and daltroban, or YM158 (30 mg/kg) significantly prolonged the onset time for asthmatic response (Fig. 1a) and significantly suppressed symptoms (Fig. 2a), whereas pranlukast alone had no effect. In groups treated with 5 mg/kg of indomethacin, administration of pranlukast, a combination of pranlukast and daltroban, or YM158 significantly prolonged the onset time for asthmatic response (Fig. 1b) and significantly suppressed symptoms (Fig. 2b), whereas daltroban alone had no effect. In these conditions, the efficacy of a combination treatment (pranlukast and daltroban) or YM158 given alone was almost the same as that of the corresponding mediator antagonist given alone. In groups treated with 1 mg/kg of indomethacin, all treated groups, including the combined treatment group, showed significant prolongation of the onset time for asthmatic response (Fig. 1c) and significant suppression of symptoms (Fig. 2c). The effects of combination treatment of both antagonists on the onset time for asthmatic response were additive effects of both by pranlukast given alone and by daltroban given alone (Fig. 1c). YM158 also showed the same potent effects on the onset times for asthmatic response and symptoms as those of a combination treatment in 1 mg/kg of indomethacin-treated conditions (Figs. 1c and 2c).

**Airway Microvascular Leakage.** The content of Evans blue dye isolated from trachea significantly increased due to antigen challenge in sensitized guinea pigs, compared with nonsensitized guinea pigs (Fig. 3). In groups in which indomethacin was not administered (Fig. 3a), and in groups in which 5 mg/kg of indomethacin was administered (Fig. 3b), pranlukast, a combination of pranlukast and daltroban, and YM158 exhibited significant suppression on antigen-evoked enhancement of microvascular leakage. When 1 mg/kg of indomethacin was given before challenge, a combination of pranlukast and daltroban, and YM158 given alone significantly suppressed extravasation, whereas pranlukast and daltroban given alone showed only the tendency to suppress extravasation (Fig. 3c). In these experiments, the weights of isolated trachea were not significantly different in all groups (data not shown).

**Combined Effect of No-Effect Doses of Single Receptor-Selective Antagonists on Antigen-Induced Asthmatic Response Associated with Both LTs and TX.** In groups treated with 1 mg/kg of indomethacin, neither pran-

---

**TABLE 1**

Lipid mediator content in sensitized guinea pig lung isolated before and 15 min after antigen challenge

<table>
<thead>
<tr>
<th>Indomethacin mg/kg i.v.</th>
<th>Mediator Contents</th>
<th>Ratio</th>
<th>p</th>
<th>TX/LTs (challenged)</th>
<th>Before/After</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td></td>
<td>Before/After</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>LTC₄/D₄/E₄</td>
<td>0.82 ± 0.12</td>
<td>7.18 ± 1.23</td>
<td>.0011</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>TXB₂</td>
<td>10.10 ± 1.08</td>
<td>71.79 ± 9.83</td>
<td>.0002</td>
<td>10.0</td>
</tr>
<tr>
<td>5</td>
<td>LTC₄/D₄/E₄</td>
<td>1.15 ± 0.40</td>
<td>33.89 ± 6.75</td>
<td>.0018</td>
<td>29.5</td>
</tr>
<tr>
<td></td>
<td>TXB₂</td>
<td>3.38 ± 0.46</td>
<td>4.91 ± 0.93</td>
<td>.2279</td>
<td>1.5</td>
</tr>
<tr>
<td>1</td>
<td>LTC₄/D₄/E₄</td>
<td>0.95 ± 0.35</td>
<td>4.50 ± 0.82</td>
<td>.0032</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>TXB₂</td>
<td>4.17 ± 0.73</td>
<td>16.37 ± 3.31</td>
<td>.0066</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Lipid mediators were measured by radioimmunoassay after the extract of lipophilic components. p values between before and after values were calculated by Student’s t test. Values are the means ± S.E. of six to nine animals.
lukast (0.3, 1, and 3 mg/kg) nor daltroban (1 and 3 mg/kg) had an effect on the onset time for asthmatic response. When 0.3 mg/kg of pranlukast and 3 mg/kg of daltroban, in doses that did not individually affect the asthmatic response, were administered concurrently, there was significant prolongation of the onset time of asthmatic response (Fig. 4). In this study, although an improvement in symptom scores was observed, the combination had no effect on extravasation of Evans blue dye (data not shown).

**Discussion**

Judging from clinical trial results of lipid mediator inhibitors such as LTD₄ receptor antagonists (pranlukast, zafirlukast) and TXA₂ receptor antagonist (seratrodast), it has been suggested that each mediator plays a role in pathogenesis of asthma, and that the predominant mediator varies from patient to patient. The existence of both responder and nonresponder to seratrodast on the hypersensitivity study (Fujimura et al., 1991) and the variability of response to zafirlukast on inhaled antigen-induced immediate decrease...
in FEV1, forced expiratory volume in one second (Dahle et al., 1994), has been reported. These clinical reports suggest that the existence of the subject heterogeneity and that each of these lipid mediator inhibitors is effective on only a part of asthmatic patients, irrespective of stage and severity in the symptom. That is, asthmatic patients may be roughly classified into LTD4- and TXA2-predominant types. On the other hand, allergic bronchoconstriction in guinea pigs is mainly mediated by histamine, and is therefore different from human bronchial asthma in which cys-LTs, TXA2, platelet-activating factor (Smith, 1991), and other mediators are thought to be important (Wenzel, 1997). Therefore, animals were pretreated with pyrilamine to eliminate the effect of histamine and to enhance lipid mediator-related components in antigen-evoked responses. From the present results, under the condition in which pyrilamine was used, the predominant mediator was TXA2 in indomethacin-nontreated guinea pigs. Thus, this condition corresponds to groups not treated with indomethacin in the present report. Pretreatment with a variety of indomethacin doses was able to control the lipid mediator contents in passively sensitized guinea pig lungs. Briefly, antigen challenge mainly increased the content of TXA2 in guinea pigs not treated with indomethacin, induced a LTC4/D4/E4-rich condition through the complete inhibition of cyclooxygenase by 5 mg/kg of indomethacin, and induced a condition in which the effects due to both mediators were almost the same using 1 mg/kg of indomethacin. To induce the same level of response in all groups, the titer of anti-BSA serum used for the sensitization was different between indomethacin-pretreated and -nontreated animals. This may be the reason that the contents of cys-LTs in the 1-mg/kg indomethacin-treated animals was lower than those in indomethacin-nontreated animals. It has been reported that TXA2 receptor antagonists are effective in guinea pigs not treated with indomethacin (Matsumoto et al., 1994), and lipooxygenase product-related agents, such as LTD4 receptor antagonists and 5-lipoxygenase inhibitors, were effective in modified models using the guinea pigs pretreated with indomethacin (Nakagawa et al., 1992; Howell et al., 1994). If it is true that asthmatic patients may be roughly classified into two types, in which either LTD4 or TXA2 is primarily responsible for asthmatic response, antiasthmatic compounds should be evaluated in several animal asthmatic models encompassing the LTD4-predominant type, the TXA2-predominant type, and a type mediated by both agents.

To determine the predominant mediator in these various conditions, the effect of pranlukast (30 mg/kg p.o.) and daltroban (10 mg/kg p.o.) was examined in doses that exhibit antagonistic activity on the onset time for asthmatic response and the severity of asthmatic symptoms. It had already been studied that these doses of pranlukast and daltroban were enough for the inhibitory effects on LTD4- or U46619 (TXA2-analog)-induced increases in airway resistance in guinea pigs, respectively (Arakida et al., 1997). As a result, it was suggested that the predominant mediator is cys-LTs in guinea pigs treated with 5 mg/kg of indomethacin, TXA2 in guinea pigs not treated with indomethacin, and both mediators (cys-LTs and TXA2) in guinea pigs treated with 1 mg/kg of indomethacin.

The additional effects of daltroban on the onset times for asthmatic response and symptom scores were not detected under cys-LTs-predominant conditions. Furthermore, the additional effects of pranlukast on the onset times for asthmatic response and symptom scores were not detected under TXA2-predominant conditions. In addition, under conditions where both mediators participated equally, the improving effects by a combination use of pranlukast and daltroban on the onset times for asthmatic response and the symptom scores were shown as more potent tendencies than those by pranlukast or daltroban given alone. Consequently, construction of a predominant mediator-controlled asthmatic model using various doses of indomethacin was successful, and further suggests activating effects...
that dual antagonism is effective on asthmatic responses irrespective of these conditions.

Extravasation of blood plasma from airway capillaries leads to the formation of mucosal edema and is thought to be one of the causal effects of airway spasm, followed by bronchial narrowing. Pranlukast inhibited the enhancement of microvascular leakage in all conditions used (Fig. 2), and these suppressive effects on the permeability of blood vessels were exhibited, although it had no effect on the onset time for asthmatic response and symptoms. Daltroban showed a tendency to suppress extravasation in animals treated with 1 mg/kg of indomethacin (Fig. 2c), but did not show suppressive effects in other conditions (Fig. 2, a and b). Under conditions in which TXA2 was predominant (Fig. 2a), the content of cys-LTs was slightly enhanced and the increase of microvascular leakage was weak. Under conditions where cys-LTs and TXA2 participate equally (Fig. 2c), the increase of microvascular leakage was the same as the LTD4-predominant condition (Fig. 2b), and this response may be partly mediated by TXA2. Because LTD4 or platelet-activating factor-induced asthmatic response was reported to be reduced by TXA2 receptor antagonists or synthetase inhibitors (Sakurai et al., 1994; Aizawa et al., 1996), the TXA2 receptor antagonism may inhibit microvascular leakage caused by cys-LTs-related conditions. In contrast, TXA2 analog-induced plasma extravasation was also reported to be partly mediated by cys-LTs and platelet-activating factor (Kawikova et al., 1996). Cys-LTs are known to increase vascular permeability by themselves (Peck et al., 1981; Rinkema et al., 1984), and, from these results, cys-LTs are considered to be the principle mediator associated with elevation of vascular permeability.

The effect of combined treatment with an LTD4 receptor antagonist and a TXA2 receptor antagonist, in doses that did not individually affect the asthmatic response, was investigated. The results showed that a significant prolongation of the onset time for asthmatic response was observed when 0.3 mg/kg of pranlukast and 3 mg/kg of daltroban were concurrently administered. Accordingly, drugs which have a dual antagonistic effect are expected to produce improvement in patients with asthma who do not respond to drugs which have only a single antagonistic effect.

The effect of YM158, a newly synthesized and orally active dual antagonist for LTD4 and TXA2 receptors, was examined on these asthmatic responses in mediator-controlled and passively sensitized guinea pigs. Because the inhibitory effects of YM158 on increase in the airway resistance induced by LTD4 or U46619 were shown to be dose-dependent when p.o. administered 1 h before LTD4 or U46619 injection, with ED50 values of 8.6 and 14 mg/kg, respectively (Arakida et al., 1997), the antagonistic activities of p.o. YM158 for LTD4 and TXA2 receptors were exhibited at the same dose range. Oral YM158 showed significant effects, approximately the same as the combination of pranlukast and daltroban on antigen-induced response under various conditions; namely, where LTD4 was predominant, TXA2 was predominant; or where both mediators participated equally.

In conclusion, this study shows that indomethacin controls the mediator-related asthmatic response, and that the main mediator-controlled models roughly reflect the classification of asthmatic patients into LT-predominant and TX-predominant types. YM158, an orally active dual antagonist for LTD4 and TXA2 receptors, is expected to have a stronger antiasthmatic efficacy in a broader class of asthmatic patients than single antagonistic drugs.

References


Send reprint requests to: Yasuhito Arakida, Inflammation Research Pharmacology Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 21, Miyuki-kuoaka, Tsukuba-shi, Ibaraki 305-8585, Japan. E-mail: arakida.yasuhito@yamanouchi.co.jp