Detection of Bowman-Birk Inhibitor and Anti-Bowman-Birk Inhibitor Antibodies in Sera of Humans and Animals Treated With Bowman-Birk Inhibitor Concentrate

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Abstract: The Bowman-Birk inhibitor (BBI) is a soybean-derived serine protease inhibitor with anticarcinogenic activities. BBI, in the form of BBI concentrate (BBIC), is currently being evaluated in clinical trials as a human cancer-preventive agent. In the present study, an enzyme-linked immunosorbent assay was used to measure BBI concentrations in serum samples collected from human subjects and animals treated with BBIC. The results demonstrate that the serum BBI concentration was higher than the baseline level for the patients after treatment with BBIC at 100–800 chymotrypsin-inhibitor units/day for 0.5, 1, 2, 4, and 6 mo. The increase in serum BBI concentration was also observed in dogs treated with BBIC at 100–1,000 mg/kg/day for 52 wk, and the increase was dose dependent. The results also indicate that anti-BBI antibodies were present in animals and the serum levels of anti-BBI antibodies increased significantly in mice treated with BBIC at 100–1,000 mg/kg/day for 15 and 26 wk. The increase in the serum level of anti-BBI antibodies in dogs treated with BBIC was not statistically significant, and no increase in the serum level of anti-BBI antibodies was observed in human subjects after BBIC treatment. These results suggest that orally ingested BBI is absorbed by human subjects and animals and that some animals develop antibodies to BBI in response to treatment with BBIC.

Introduction

The Bowman-Birk inhibitor (BBI) is a soybean-derived serine protease inhibitor that inhibits trypsin- and chymotrypsin-like proteases, such as trypsin, chymotrypsin, cathepsin G, elastase, and chymase (1). BBI is also an anticarcinogenic agent that inhibits malignant transformation in vitro and suppresses cancer development in several organ systems and animal species (1–3). Epidemiological data have shown that high levels of soybean consumption are correlated with low incidence rates of colon, breast, and prostate cancers in human populations (4). These observations suggest that BBI could be a useful agent for cancer prevention in humans. BBI, in the form of BBI concentrate (BBIC), is currently being evaluated as a cancer-preventive agent. Studies in animals have demonstrated that BBIC is well tolerated by dogs (5), rats (6), and mice (7) at up to 1,000 mg/kg/day without significant drug-induced toxicity (5–8). Human studies have also shown that BBIC is not toxic at ≤800 or 1,066 chymotrypsin-inhibition (CI) units/day (9–12); 1 CI unit is defined as the amount of protease inhibitor needed to inhibit 1 mg of bovine pancreatic α-chymotrypsin (13). In a Phase IIa oral cancer prevention trial in patients with oral leukoplakia, treatment with BBIC at 200–1,066 CI units/day for 1 mo resulted in a dose-dependent decrease in oral lesion size (11). In a Phase I trial of BBIC in patients with benign prostatic hyperplasia, treatment with BBIC at 100–800 CI units/day for 6 mo resulted in reductions in serum prostate-specific antigen levels and prostate volume (12).

In toxicity studies and clinical trials to evaluate BBI as a cancer-preventive agent, a method for detecting BBI and its metabolites in body fluids is essential for monitoring the BBI exposure. We previously demonstrated that BBI can be detected in the urine of human subjects by an immunoassay method using a monoclonal antibody (MAb) against reduced BBI (10–12,14). In the present study, we measured BBI concentrations in serum samples collected from human subjects and animals treated with BBIC. The results demonstrated an increase of serum BBI concentration in the human subjects and dogs after BBIC. The results also showed that anti-BBI antibodies were present in mouse sera and that the serum titers of the anti-BBI antibodies increased significantly in the mice treated with BBIC.

Materials and Methods

Antibodies and Chemicals

The MAb 5G2 was prepared and characterized as previously described (15). BBI was purchased from Sigma Chemi-
Serum Samples From Animals With BBIC analyses. Serum samples from animals in a 6-mo BBIC toxicity study, which has been described elsewhere (13). Horseradish peroxidase-conjugated goat anti-mouse IgG was purchased from Southern Biotechnology (Birmingham, AL). Horseradish peroxidase-conjugated rabbit anti-dog IgG and rabbit anti-human IgG were purchased from Sigma Chemical.

Serum Samples From Patients Treated With BBIC

Human serum samples used in the present study were obtained from 19 male patients enrolled in a 6-mo Phase I BBIC clinical trial, which has been described in detail elsewhere (9,12). In that trial, placebo or BBIC tablets were administered orally at 100, 200, 400, or 800 mg/day for 6 mo. Nonfasting blood samples were collected from patients before and during the 6-mo treatment period. Serum was separated from the whole blood and stored at −20°C before analyses.

Serum Samples From Animals With BBIC

Mouse serum samples used in the present study were obtained from animals in a 6-mo BBIC toxicity study, which was performed by D. G. Serota (Laboratory Study Identification No. 560-057) and reported to the National Cancer Institute, Division of Cancer Prevention, Chemoprevention Branch on 3 January 2000 (7). In this study, 100 B6C3F1 mice were divided into 4 groups of 25 animals per group, and BBIC was orally administered at 0, 100, 500, or 1,000 mg/kg/day for 6 mo. During the 6-mo treatment period, the animals were fed a diet that was modified from Certified Rodent Diet 5002 (PMI Feeds, St. Louis, MO) by replacement of the soybean component with an equivalent meat by-product component. Blood samples were collected from the first 10 animals of each gender and treatment group at Week 15 and at the termination of the study. Dog serum samples used in the present study were obtained from animals in a 1-yr BBIC toxicity study performed by D. G. Serota (Laboratory Study Identification No. 560-058) and reported to the National Cancer Institute, Division of Cancer Prevention, Chemoprevention Branch on 27 January 2000 (8). In this study, BBIC was orally administered to 16 male and 16 female beagle dogs at 0, 100, 500, or 1,000 mg/kg/day for 1 yr. During the 1-yr treatment period, the animals were fed modified Certified Canine Diet 5LC7 (PMI Feeds, Ft. Wayne, IN); methods for the production of BBIC have been described elsewhere (14). Horseradish peroxidase-conjugated goat anti-mouse IgG was applied to 96-well polystyrene plates for 1 h to block nonspecific binding sites on the plates, and then frozen and stored at −20°C before use. To perform the experiments, 50 µl of MAb 5G2 (diluted 1:2,000 in BSA-PB) were mixed with an equal volume of the serum to be tested in a well on the 96-well plates precoated with rBBI and incubated for 30 min. The color development was stopped by addition of 100 µl of 1.0 N HCl to each well. All incubations were carried out at room temperature, and the plates were read at a wavelength of 450 nm with a PowerWave 340 microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, VT). In each experiment, the serum BBI concentrations were estimated using a standard curve generated in the same experiment.

Detection of Anti-BBI Antibodies in Sera

The anti-BBI antibodies in the serum samples were also detected by the ELISA method. To carry out the experiments, 10 µl of each serum sample were diluted with 40 µl of BSA-PB in a well on the 96-well plates precoated with rBBI and incubated for 90 min. For negative controls, BSA-PB was added in place of serum sample. The plates were then washed three times with water and further incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (50 µl/well) for 1 h. The plates were again washed three times with water and incubated with tetramethylbenzidine substrate for 30 min. The color development was stopped by addition of 100 µl of 1.0 N HCl to each well. All incubations were carried out at room temperature, and the plates were read at a wavelength of 450 nm with a PowerWave 340 microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, VT). In each experiment, the serum BBI concentrations were estimated using a standard curve generated in the same experiment.

Data and Statistical Analysis

The standard curve utilized for estimating the serum BBI concentrations was established by semilogarithmic regression analysis using the logarithm of BBI concentration as the
independent variable and the absorbance at 450 nm as the dependent variable. The BBI concentrations (ng/ml) in the serum samples were determined using the standard curves generated as part of each experiment. The group means of the serum BBI concentrations and the anti-BBI antibody levels were analyzed by the regression analysis or one-way analysis of variance (ANOVA) followed by the Student’s t-test and/or Tukey’s test using Prism version 2.0 statistical software (GraphPad Software, San Diego, CA).

Results

We previously demonstrated that MAb 5G2, which was developed against rBBI (15), can be utilized to detect BBI in the urine of human subjects after oral administration of BBIC (10–12) or ingestion of BBI-containing soy milk (14). The present study involves measurements of BBI concentrations in sera of human subjects and animals treated with BBIC. BBI concentrations in human serum were determined for 10 patients who were treated with placebo (n = 4) or BBIC at 100 (n = 3), 200 (n = 1), 400 (n = 2), or 800 (n = 4) CI units/day for 6 mo. There are small but varying numbers of missing data points because of inadequate or unavailable samples. Given the small number of patients, those in the four BBIC dose groups were combined into a single BBIC treatment group for the purpose of statistical analysis. Before BBIC treatment, the baseline values for the serum BBI concentration measured in the 10 patients assigned to the BBIC treatment group and the 4 patients assigned to the placebo control group were equivalent to 140 ± 155 and 97 ± 99 (SD) ng/ml, respectively. The baseline values of serum BBI concentrations varied substantially among the individuals, as indicated by the relatively large standard deviations. The high baseline value for the BBIC treatment group was entirely due to one patient whose serum BBI concentration (536–685 ng/ml before and after BBIC treatment) was exceptionally and persistently higher than that of all other patients enrolled in the study. Without this patient, the baseline value for the BBIC treatment group was 96 ± 72 ng/ml of BBI, which was very close to that of the placebo control group. At 0.5, 1, 2, 4, and 6 mo of BBIC treatment, the average serum BBI concentration for the BBIC treatment group was 66.37%, 56.05%, 51.24%, 25.70%, and 13.98% higher than the baseline value (Fig. 1). The serum BBI concentrations at 0.5 or 1 mo of BBIC treatment were significantly higher than the baseline value (P ≤ 0.025, paired Students’ t-test). In contrast, the serum BBI concentrations measured for the control group during the 6-mo placebo treatment period were mostly below the baseline value and did not differ significantly from the baseline value (P > 0.44, paired Student’s t-test) at any of the five time points (Fig. 1).

The BBI concentrations in dog serum were determined in 16 male and 16 female beagle dogs that had been treated orally with BBIC at 0 (carrier control), 100, 500, or 1,000 mg/kg/day for 13 or 52 wk. Before BBIC administration, the baseline values of serum BBI concentration were not significantly different among the dogs assigned to the four treatment groups (P > 0.80, 1-way ANOVA, data not shown).

Figure 1. Serum Bowman-Birk inhibitor (BBI) concentrations in patients treated with BBI concentrate (BBIC) or placebo tablets for 6 mo. Placebo or BBIC tablets were orally administered to patients with benign prostatic hyperplasia twice a day at 100–800 chymotrypsin-inhibitor units/day for 6 mo. Blood was drawn from patients before and during treatment. Serum BBI concentrations were determined by enzyme-linked immunosorbent assay (ELISA).
The serum BBI concentrations were not significantly different for the dogs of the four treatment groups after 13 wk of BBIC treatment ($P > 0.10$, 1-way ANOVA, data not shown).

After 52 wk of BBIC treatment, the serum BBI concentrations were ~25% ($P = 0.107$), 23% ($P < 0.05$), and 49% ($P < 0.001$) higher for the animals treated with BBIC at 100, 500, and 1,000 mg/kg/day, respectively, than for the control group (Table 1). The increase in the serum BBI concentrations was dose dependent (Fig. 2).

We also attempted to measure the BBI concentrations in the sera of mice treated with BBIC at 0 (carrier control), 100, 500, and 1,000 mg/kg/day for 15 or 26 wk. In the inhibitory ELISA used for this study, the absorbance reading was inversely correlated with the logarithm of BBI concentration (Fig. 3), and the highest absorbance reading was expected for the negative control, which contained no free BBI antigen to inhibit the binding of MAb 5G2 to the immobilized BBI antigen. Antibodies bound to rBBI antigen were detected with a horseradish peroxidase-conjugated goat antibody against mouse IgG. ELISA, enzyme-linked immunosorbent assay; $n$, number of animals.

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### Table 1. BBI Concentrations in Sera From Dogs Treated With BBIC for 52 Weeks

<table>
<thead>
<tr>
<th>BBIC Dose, mg/kg/day</th>
<th>$n$</th>
<th>BBI Conc., ng/ml</th>
<th>Increase, %</th>
<th>$P$ (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8</td>
<td>202.53 ± 61.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>7</td>
<td>253.34 ± 96.94</td>
<td>25.08</td>
<td>0.107</td>
</tr>
<tr>
<td>500</td>
<td>8</td>
<td>248.64 ± 65.14</td>
<td>22.77</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>1,000</td>
<td>8</td>
<td>301.48 ± 85.71</td>
<td>48.86</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*a*: Serum samples from dogs treated with Bowman-Birk inhibitor (BBI) concentrate (BBIC) at 0 (control), 100, 500, or 1,000 mg/kg/day for 52 wk were mixed with monoclonal antibody 5G2 solution and incubated in microwell plates precoated with reduced BBI (rBBI) antigen. Antibodies bound to rBBI antigen were detected with a horseradish peroxidase-conjugated goat antibody against mouse IgG.

*b*: Values are means ± SD.

### Table 2. Absorbance Data From an ELISA Experiment Intended to Measure Mouse Serum BBI Concentrations

<table>
<thead>
<tr>
<th>Time of Blood Collection</th>
<th>BBIC Dose, mg/kg/day</th>
<th>Absorbance at 450 nm</th>
<th>$n$</th>
<th>$P$ (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 15</td>
<td>0</td>
<td>0.903 ± 0.288</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.324 ± 0.564</td>
<td>10</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>1.440 ± 0.546</td>
<td>9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>1.303 ± 0.530</td>
<td>10</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Week 26</td>
<td>0</td>
<td>0.947 ± 0.476</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.663 ± 0.491</td>
<td>10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>1.300 ± 0.579</td>
<td>10</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>1.388 ± 0.474</td>
<td>10</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*a*: Serum samples from mice treated with BBIC at 0 (control), 100, 500, or 1,000 mg/kg/day for 15 or 26 wk were mixed with monoclonal antibody 5G2 solution and incubated in microwell plates precoated with rBBI antigen. Antibodies bound to rBBI antigen were detected with a horseradish peroxidase-conjugated goat antibody against mouse IgG. ELISA, enzyme-linked immunosorbent assay; $n$, number of animals.

*b*: Values are means ± SD.
Figure 4. Detection of anti-BBI antibodies in serum from mice treated with BBIC. BBIC was orally administered to a total of 100 B6C3F1 mice at 0 (carrier control), 100, 500, or 1,000 mg/kg/day for 26 wk (25 mice for each dose group). Blood was drawn from first 10 animals in each dose group after 15 and 26 wk of BBIC treatment, and serum levels of anti-BBI antibodies were determined by ELISA. P values represent comparison between control group and each treatment group (1-way analysis of variance). 

Discussion

MAb 5G2 was produced against the reduced form(s) of the BBI molecules (15) and was able to detect BBI in human urine samples after BBIC administration (10–12) or ingestion of BBI-containing soy milk (14). The present study has demonstrated that this antibody can also be used to measure BBI in human and dog serum samples. In human subjects, the serum BBI concentration varied greatly, even before BBIC was administered. This is reflected in the large coefficients of variation (SD ÷ mean) for the serum BBI concentration, which are 1.021 (99 ÷ 97) and 1.107 (155 ÷ 140), respectively, for the patients in the placebo control group and the BBIC treatment group. The large individual variations in the serum BBI concentration could have been caused by the difference in diets consumed by the patients, because BBI is present at various levels in soybean-containing foods. The large individual variations could also have been caused by the difference in the time between BBIC administration and blood sample collection, because the length of time between BBIC administration and blood sample collection was not controlled in the clinical trial. Because of the large individual variation among the patients, the serum BBI concentrations measured in individual patients after BBIC treatment were compared with the pretreatment serum BBI concentrations in the same patients by the paired Student’s t-test to avoid masking of treatment-associated changes by the individual variations. The results demonstrated that the serum BBI concentration was increased significantly in patients after 2 and 4 wk of BBIC treatment. The average serum BBI concentration for patients after 2, 4, or 6 mo of BBIC treatment was also higher than the baseline value, although the magnitudes of increase in the serum BBI concentration were not as large as that observed after 2 or 4 wk of BBIC treatment. These results are consistent with the previous observation that BBI levels in urine were increased in these patients after BBIC treatment (12). These findings suggest that BBI was absorbed after oral ingestion.

In contrast to the large individual variation observed in the human subjects, the individual variation for the serum BBI concentration in dogs before BBIC treatment was small, as reflected by the smaller coefficient of variation (62 ÷ 203 = 0.305). This is not surprising, because the dogs were fed the same food before and during the study, and the blood samples were collected at the same time. The serum BBIC concentration displayed a dose-dependent increase in dogs after 52 wk of treatment with BBIC. Such an increase was not observed in the animals after 13 wk of BBIC treatment. This is different from the time course observed in the human BBIC clinical trial, in which the increase in the serum BBI concentration was most significant after 2–4 wk of BBIC treatment and then gradually diminished during the remaining 5 mo of BBIC treatment. The cause for such a difference is not known.

In the present study, the serum BBI concentration in mice could not be measured by the inhibitory ELISA method because of the existence of anti-BBI antibodies in the mouse serum. The binding of the anti-BBI antibodies to the immobilized rBBI antigen made it impossible to measure the serum BBI concentration in mice by the inhibitory ELISA method. It is unlikely that this problem can be overcome by
changing the design of the ELISA method, because the anti-BBI antibodies in the mouse serum will most likely mask the BBI antigen and prevent its detection by other immunoassay methods. The levels of anti-BBI antibodies were significantly higher in the mice treated with BBIC than in the mice treated with empty capsules, suggesting that the mice produced anti-BBI antibodies in response to the BBIC treatment. The increase in serum levels of anti-BBI antibodies in dogs after BBIC treatment was not significant, and no change in the serum levels of anti-BBI antibodies was observed in human patients after BBIC treatment.

The different antibody responses to orally ingested BBI in dogs and mice could reflect a species difference, because the dogs and mice were treated with BBIC at the same doses in relation to their body weight. It is also possible that the difference in the anti-BBI antibody responses between these two species is related to their prior exposure to BBI in the diet. In this study, the dogs and mice were fed diets devoid of soybean products during the BBIC treatment periods; however, the diets used before the treatment periods were unmodified Certified Canine Diet and Certified Rodent Diet, which contain soybean as a major ingredient. Because the soybean content in the unmodified Certified Rodent Diet is ~40% higher than in the unmodified Certified Canine Diet (personal communication, Dr. Dorrance Haught, Purina Mills), the antibody response to orally ingested BBI observed in mice could be a result of the relatively higher prior exposure to BBI in the diet. The lack of antibody response to orally ingested BBI in human patients may or may not be due to a species difference, because the highest BBIC dose received by the patients was only equivalent to ~10% of that received by the dogs and mice. The average level of soybean consumption in the US population has been estimated to be 2.5 g/capita/day (18) or 0.16% assuming a normal daily dietary intake of 1,600 g, which is about two orders of magnitudes below the levels of soybean constituents in the canine and rodent diets and is unlikely to be sufficient to elicit an anti-BBI antibody response.

The development of antibodies to orally ingested proteins has been reported previously for proteins other than BBI. For instance, orally administered bovine milk lactoferrin was shown to induce the production of anti-lactoferrin antibodies in female BALB/c mice (19). In germ-free rats fed ovalbumin-containing food, the levels of antiovalbumin antibodies increased (20). In young piglets abruptly weaned onto soy food at 3 wk of age, antibodies against soybean protein were produced, and the serum levels of the anti-soybean protein antibodies were almost comparable to those induced by immunization with soybean protein in adjuvant at 7 wk (21).

The levels of the anti-BBI antibodies in the mice treated with BBIC were relatively low, and a relatively high concentration (20% in this study) of the mouse serum was needed to demonstrate the existence of the anti-BBI antibodies. This is consistent with the observations on the antibody responses to other orally ingested proteins. For example, the antibody response to orally ingested ovalbumin was very weak and was mostly IgM (20). In the piglets primed by feeding with soybean food, no further increase in the levels of antibodies occurred after subsequent immunization with soybean proteins, suggesting the development of specific unresponsiveness to soy proteins in neonates fed soybean food (21). Given that the levels of the anti-BBI antibodies were relatively low in the mice treated with BBIC and were below the detection limit in human patients after BBIC treatment, it is not expected that oral administration of BBIC will result in clinically meaningful complications related to an immune response to BBI antigen.

In previous studies, BBI was shown to be effective in suppressing cancer development in the lung (22), liver (23), and colon (24) of mice exposed to 3-methylcholanthrene (22) or dimethylhydrazine (23,24). The results of the present study have demonstrated that mice developed antibodies against BBI after 15 wk of treatment with BBIC. It is not clear why BBI is effective as an anticarcinogenic agent in a species of animals that develop anti-BBI antibodies after BBI exposure. One possible explanation is that the levels of anti-BBI antibodies developed in the mice might be too low to completely neutralize the anticarcinogenic effect of BBI. Another possible explanation is that BBI might have already exerted its anticarcinogenic effect by the time the anti-BBI antibodies were developed. If this is the case, a diminished effect of BBI could be expected in mice with prior BBI exposure. Because the anti-BBI antibodies were not detected in human patients after 6 mo of treatment with BBIC, the anticarcinogenic effect of BBI is not expected to be diminished in humans by prior BBI exposure.

Acknowledgments and Notes

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