PRODUCTION OF CROSS-REACTIVE AUTOANTIBODY BINDING TO BOVINE SERUM ALBUMIN IN THE D-GALACTOSE-INDUCED AGING MOUSE MODEL

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ABSTRACT
The D-galactose (D-gal)-induced animal model, generated by repeated subcutaneous D-gal injections over approximately 6 weeks, has been frequently used for diabetes and aging research. However, little research has investigated the direct correlation between D-gal and autoantibody formation despite several reports on diabetes-and aging-related autoantibodies. The purpose of this study was to determine whether repetitive injection of D-gal can induce autoantibody production in mice. First, we used Bovine Serum Albumin (BSA) and Advanced Glycation End products (AGE)-BSA as the test antigens. The immunoreactivity of serum samples from mice treated with D-gal for 6 weeks was evaluated using Enzyme-Linked Immunosorbent Assay (ELISA). We found that serum samples of D-gal-treated mice had significantly high antibody titers against both BSA and AGE-BSA. Furthermore, the result showed that aminoguanidine treatment, an AGE inhibitor tended to decrease this immunoreactivity. The results of competitive inhibition ELISA using BSA and AGE-BSA as the competitors suggested that the serum samples from D-gal-treated mice contained antibodies not only against BSA but also specific to AGE-BSA. To assess whether the immunoreactivity against BSA is comparable to that against Mouse Serum Albumin (MSA), we examined the reactivity of D-gal-induced antibodies against MSA. Unexpectedly, D-gal-induced antibodies did not react with MSA. This suggests that the production of antibodies by D-gal is in response to an unknown antigen(s), aside from MSA, in mice and that this unknown antigen(s) may share similar sequences or three-dimensional structures with BSA.

Keywords: D-Galactose-Induced Aging Model, Advanced Glycation End Products, Autoantibody, Bovine Serum Albumin, Mouse Serum Albumin

1. INTRODUCTION
Advanced Glycation End products (AGEs) are formed in vivo by the Maillard reaction, a non-enzymatic reaction of proteins or lipids with carbohydrates (Horieuchi et al., 1991). Many studies on AGEs have been conducted in diabetes models (Berg et al., 1997; Rumble et al., 1997; Cooper, 2004), with chronic high blood sugar levels and in aging models (Shuvaev et al., 2001; Odetti et al., 2005; Luevano-Contreras and Chapman-Novakofski, 2010), each displaying gradual accumulation of AGEs. For example, AGEs such as N-(carboxymethyl) Lysine (CML) and pentosidine have been found to accumulate on proteins of the skin and lens at accelerated rates in diabetic and aged tissues (Dyer et al., 1993; Handa et al., 1999). In this regard, the D-gal-induced aging model was established by artificially inducing high levels of AGEs through repetitive injections of highly concentrated carbohydrates (Song et al., 1999).

The D-gal-induced aging mouse model was produced by consecutive subcutaneous D-gal injections over approximately 6 weeks (Song et al., 1999). This animal model shows accelerated aging in the brain, ear, kidney,
2.1. Animals and D-Gal Treatment

ICR female mice aged 8-10 weeks were used for this study. The mice were provided drinking water ad libitum and a normal diet and kept under a 12-h light-dark cycle at 24±1°C and 50% humidity. Mice were randomly divided into three groups containing five mice each. After a 1-week adaptation period, mice from each group were injected subcutaneously daily with 1,000 mg kg⁻¹ D-gal or vehicle (0.9% saline) as a control for approximately 6 weeks (Park and Choi, 2012). One of the D-gal-treated groups was continually administered 0.1% aminoguanidine, an AGE inhibitor, in the drinking water. All animal studies were conducted in compliance with the guidelines for the Care and Use of Research Animals established by the Animal Studies Committee of Dankook University.

For blood plasma preparation, whole blood was obtained by incising the tail vein with a sharp surgical blade and then centrifuged at 1,500×g for 15 min at room temperature. The plasma was aliquoted and frozen at -80°C until use.

2.2. AGE-BSA Production

AGE-BSA was prepared as described previously (Wang et al., 2011). In brief, 50 mg mL⁻¹ fatty acid-free BSA (Sigma-Aldrich) was incubated with 0.5 M glucose in 10 mM PBS containing 0.5 mM EDTA, 100 units mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin, pH 7.4 at 37°C for 12 weeks under sterile conditions. Nonglycated BSA was prepared under the same conditions but without glucose. After incubation for 12 weeks, unincorporated sugar was removed by dialysis against PBS for 24 h. The prepared AGE-BSA and BSA were sterilized by ultrafiltration and stored at -70°C until use.

2.3. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed in 96-well polystyrene plates. The plate was coated with 100 µL of the 20 µg mL⁻¹ BSA, AGE-BSA, or MSA (Sigma Aldrich) in 0.05 mol L⁻¹ carbonate-bicarbonate buffer, pH 9.6. The plates were incubated for 2 h at 37°C or overnight at 4°C. Unbound antigen was washed three times with PBS-T (20 mM PBS, pH 7.4, 0.05% Tween-20) and the unoccupied sites were blocked with 2% nonfat milk in PBS-T for 1 h at room temperature. After incubation, the plates were washed again three times with PBS-T. To quantitate the antibodies in serum, serial two-fold dilutions were used ranging from 1:400 to 1:3200. Diluted test serum was added to the antigen-coated wells and re-incubated for 2 h at room temperature or overnight at 4°C. Bound IgG antibodies were measured using the ELISA colorimetric detection kit (KPL, BluePhos® Microwell Phosphatase Substrate System). The absorbance of each well was measured at 595 nm with an automatic microplate reader and the mean of duplicate readings for each sample was recorded.

For competition ELISA, the plate was coated with 20 µg mL⁻¹ AGE-BSA. Varying concentrations (0, 10, 100, 1000 µg mL⁻¹) of BSA or AGE-BSA were pre-incubated...
with 400-fold diluted mouse serum for 2 h at room temperature and the mixture was subsequently added to the antigen-coated plates. The remaining steps were the same as that for standard ELISA. Percentage inhibition was calculated as 1- (A inhibited/A uninhibited) × 100.

2.4. Statistical Analyses

Statistical differences among groups were analyzed using a one-way Analysis of Variance (ANOVA). Data are shown as the means ± Standard Error of the Mean (SEM) and significance was defined as p<0.05.

3. RESULTS

3.1. Antibody Production Against BSA and AGE-BSA in D-Gal Treated Mice

The immunoreactivity against BSA and AGE-BSA was tested using ELISA. Blood samples were serially diluted from 1:400 to 1:3200. The antibody titers against BSA and AGE-BSA were significantly high and similar for both antigens (Fig. 1). In contrast, decreased antibody titers were detected in serum samples from mice treated simultaneously with D-gal and aminoguanidine. These results showed that antibodies were produced against BSA and AGE-BSA in the serum of D-gal-treated mice and that the production of antibodies may be due to the formation of AGEs.

3.2. Inhibition of Antibody Binding to AGE-BSA by Pretreatment of Serum with BSA or AGE-BSA

To investigate whether an antibody specific to AGE-BSA exists among those induced by treatment of mice with D-gal, we performed competitive inhibition ELISA. We used AGE-BSA as the antigen and pre-incubated the serum samples with varying concentrations of BSA or AGE-BSA as the first antibody. Inhibition rates were markedly higher with pre-incubation of AGE-BSA (27 and 47%) compared with that of BSA (5 and 27%) at 10 and 100 µg mL⁻¹, respectively (Fig. 2). The results indicate the existence of a specific antibody for AGE-BSA among those induced by D-gal treatment.

3.3. D-Gal-Induced Antibodies do not Cross-React with MSA

To evaluate whether the antibodies induced by D-gal treatment interact with not only BSA but also MSA, we performed ELISA using MSA as the ELISA antigen. As shown in Fig. 3, the antibodies showed no reactivity against MSA.

![Graph showing immunoreactivity against AGE-BSA and BSA](image_url)
Fig. 2. Inhibition of D-gal-induced antibody binding to the AGE-BSA antigen using free BSA or AGE-BSA as the competitor. The microtiter plates were coated with AGE-BSA (20 µg mL⁻¹). Inhibition of D-gal-induced antibody binding to AGE-BSA was measured by pre-incubation of the serum with competitors (free BSA or AGE-BSA) at the indicated concentration. The percent inhibition was calculated as described in the Materials and Methods. Data represent the means ± S.E.M. of five mice per group. Different superscripts represent significantly different values (p<0.05).

Fig. 3. Immunoreactivity of the serum of D-gal-treated mice to Mouse Serum Albumin (MSA) and BSA. Serum samples from each mouse group were diluted as indicated and tested for immunoreactivity against BSA using ELISA. Data represent the means ± S.E.M. of five mice per group.

4. DISCUSSION

Autoantibody formation is a critical cause of autoimmune diseases and many autoantibodies have been associated with diabetes and aging (Wasserfall and Atkinson, 2006; Kay, 2013). It occurs when an antibody against invasive harmful bacteria or viruses interacts with self-proteins that have similar epitopes or those that are modified. As an example of the latter, recent study have suggested the possibility that AGEs produced by non-enzymatic reactions in vivo serve as antigens for autoantibody formation (Turk et al., 2001). In this respect, our results showed another example of autoantibody production caused by excessive AGE...
formation in vivo, although the protein antigen(s) was not identified by this study.

Our first hypothesis was that the production of autoantibodies against MSA, which is readily glycated and a precursor for AGE formation, occurs in D-gal-treated mice. We examined the formation of autoantibodies against BSA or AGE-BSA first because we have previously isolated AGE-BSA. We found high antibody titers against BSA and AGE-BSA in serum samples of D-gal-treated mice and this antibody production was presumed to relate to AGE formation, according to the results obtained from the aminoguanidine experiment (Fig. 2). The partial inhibitory effect on antibody production by aminoguanidine may be due to the higher concentrations of D-gal used in this study compared with previous reports (Song et al., 1999).

In addition, the results of the competitive inhibition ELISA using serum samples pre-incubated with free BSA or AGE-BSA showed that the antibodies were produced not only in response to BSA but also AGE-BSA. It appears that these antibodies were hapten- and carrier-protein-specific when immunization was performed with hapten-conjugated proteins. Recent studies have shown that AGEs possess immune potential and behave as do haptens (Bozhinov et al., 2012).

Refuting our first hypothesis, the antibodies did not at all cross-react with MSA (Fig. 3). The results suggest the possibility that an unknown protein susceptible to AGE(s), aside from MSA, serves as an epitope(s) for antibody production in D-gal-treated mice and that this epitope(s) corresponds to an epitope(s) of BSA. Previous studies demonstrated that production of BSA antibodies in vivo is due to the consumption of cow's milk, which contains BSA (Ferguson, 1977; Helms and Rieger, 1987; Lerner et al., 1989). In addition, several reports suggested that the potential for BSA antibody formation is related to the onset of type 1 diabetes, during which BSA antibodies share a common epitope with the pancreatic islet cell surface protein ICA69 (Yokota et al., 1990; Pietropaolo et al., 1993). However, other studies showed contradictory results in regard to the relationships between BSA antibody and milk consumption or type 1 diabetes onset (Ronningen et al., 1998; Birgisdottir et al., 2002) currently, most researchers agree that there is no direct correlation between them (Scott et al., 1996). Nonetheless, the mechanism of BSA antibody production in vivo remains unknown. In this respect, our discovery of a D-gal-induced BSA antibody that does not exist and cannot be introduced in mice may provide new insight into this mechanism.

5. CONCLUSION

The present study found for the first time that BSA antibody production in the D-gal-induced aging mouse model. Furthermore, from the results of D-gal-induced antibodies did not react with MSA, this suggests that the production of antibodies by D-gal is in response to an unknown antigen(s), aside from MSA, in mice and that this unknown antigen(s) may share similar sequences or three-dimensional structures with BSA. Further studies against synthetic peptides derived from the sequence of BSA, which are not in MSA are needed to identify an unknown antigen(s).

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