

Original Articles

Anti-fungal Cell Wall β -glucan Antibody in Animal Sera

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β -Glucan is a major component of the cell walls and pathogen-associated microbial patterns of fungi. We previously reported the presence of an antibody which reacts to β -glucan, anti- β -glucan (BG) antibody, in human sera. In livestock and domestic pets, the antibody's response to fungal cell wall β -glucan is little understood. In this study, we examined the existence and reactivity of anti-BG antibody in various animal species.

We demonstrated the presence of the anti-BG antibody in each animal's serum. Individual differences in the titer existed. The antibody was highly reactive to *Candida* solubilized cell wall β -glucan (CSBG) while reacting little to grifolan (GRN) from *Grifola frondosa*. This suggested that the anti-BG antibody interacted with fungal cell wall β -glucan and participated in the immune-response to pathogenic fungi.

Key words : fungal infection, β -glucan, anti- β -glucan antibody, humoral immunity

Introduction

In animals as in humans, deep-mycosis as an opportunistic infection frequently becomes a clinical problem despite improvements in veterinary medical technology and prevention. Deep-mycoses such as aspergillosis, candidiasis, blastomycosis, histoplasmosis and cryptococcosis have been reported in farm animals such as cows, horses and pigs and pets such as dogs and cats¹⁾. The diagnosis of these diseases is often based on clinical signs, and on histological and mycological findings.

Approximately 80% of the fungal cell wall is composed of polysaccharides, the main constituents being β -glucan, mannan, galactomannan and chitin²⁾. The cell wall also contains protein and a small amount of lipid. Fungal cell wall β -glucan is composed of β -1,3- and/or β -1,6-glycosyl linkage. β -1,3-glucan forms a rigid skeleton and imparts physical strength to the fungal cell wall (excluding Zymomycetes) but is not found in bacteria. β -1,3-glucan is the target of the anti-fungal drug echinocandin³⁾. β -1,6-Glucan plays a role in the connection to other cell-wall components such as

mannoprotein and β -1,3-glucan. The branching ratio and length of β -1,6-glucan differ among fungi. The primary structure of a soluble β -glucan prepared from mushrooms consists of units of six-branched β -1,3-glucan, whereas a particulate glucan prepared from yeast is made up of slightly branched long β -1,6-glucan and β -1,3-glucan segments. It was reported that β -1,3-glucan showed various biological activities triggering the activation of complement and the production of inflammatory mediators such as leukotriene and TNF- α ⁴⁾. The difference in primary structure influenced physical properties, interaction with the receptor and biological activities⁵⁾. It is possible that β -1,3- and β -1,6-glucan has some influence on the immune response and inflammatory reactions of the host.

Many researchers have examined the fungus-host relationship and anti-fungal immune mechanisms using mouse models. Many of the pattern recognition receptors involved in the innate immune response to fungi, such as the Toll-like receptor, dectin-1 and so on, have been identified⁶⁾. There are few reports on a specific antibody to β -glucan. It is generally accepted that β -glucans are not a good antigen for inducing a specific response. Indeed, the establishment of a mushroom β -glucan-specific monoclonal antibody has been difficult to achieve⁷⁾. However, an anti- β -glucan (BG) antibody was detected in sera from human

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Table 1. List of polysaccharides and their characteristics

Source	Abbreviation	Main components	References/remarks
<i>Agaricus brasiliensis</i>	AgHWE	β -1,6-glucan with β -1,3-glucan segment	Ohno N et al., 2001 ¹⁴⁾
<i>Grifola frondosa</i>	GRN	β -1,6-branching β -1,3-glucan	Ohno N et al., 1986 ¹³⁾
<i>Candida albicans</i>	CSBG	β -1,3-linked β -1,6-glucan	Ohno N et al., 1999 ¹¹⁾
<i>Aspergillus niger</i>	ASBG	β -1,3-glucan with β -1,6-glucan segment	Ishibashi K et al., 2004 ¹²⁾
<i>Saccharomyces cerevisiae</i>	Y-Man	mannan	Yeast mannan prepared from <i>Saccharomyces cerevisiae</i> . purchased from Nakalai Tesque, Ltd.

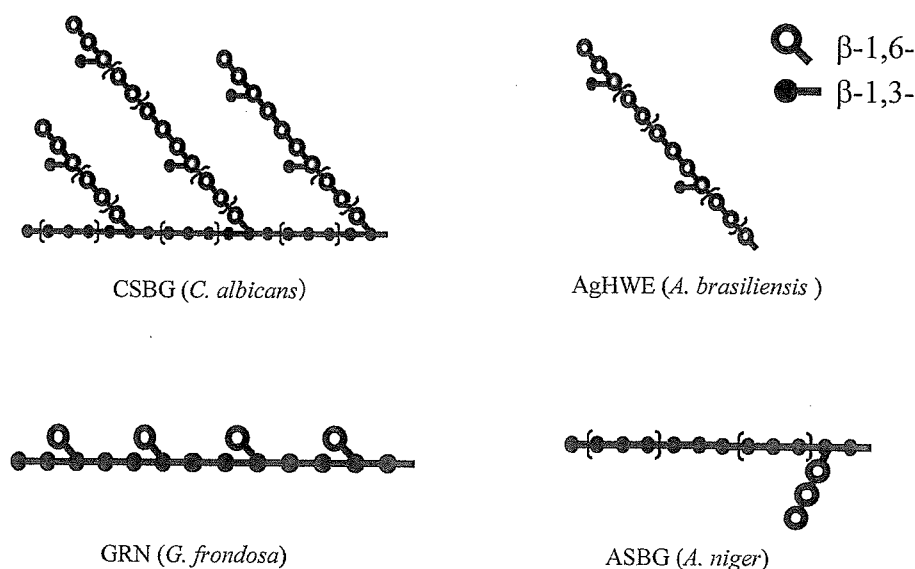


Fig. 1. Structure and characteristics of polysaccharides.

volunteers⁸⁾. The anti-human BG antibody in a globulin preparation was highly reactive to pathogenic fungal β -glucan and much less reactive to six-branched β -1,3-glucan prepared from mushroom. The titer of anti-BG antibody differed significantly in the sera from human volunteers. Human anti-BG antibody reacted more to β -1,6-glucan chains than β -1,3-glucan chains⁹⁾. The antibody titer fluctuated in patients with deep mycosis, whose sera were β -1,3-glucan-positive. Also, in the sera of most mice housed in specific pathogen-free animal facilities, small amounts of anti-BG antibody was detected¹⁰⁾. In DBA/1 and DBA/2 mouse strains, however, the antibody was significantly detected. The antibody mainly reacted to β -1,3-glucan chains. The titer and reactivity of the anti-BG antibody would differ according to species.

The humoral response to fungal cell wall β -glucan in livestock and domestic pets is little understood. In this study, we examined the presence and reactivity of the anti-BG antibody in different animal species.

Materials and Methods

Materials

Aspergillus niger NBRC6342 and *Candida albicans* NBRC 1385 were purchased from NITE Biological Resource Center (Chiba, Japan), maintained on Sabouraud agar (Becton Dickinson, MD, USA.) at 25°C, and transferred once every three months. The sodium hypochlorite solution and sodium hydroxide were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). The distilled water was from Otsuka Co., Ltd. (Tokyo, Japan). *Candida albicans* and *Aspergillus niger* solubilized cell wall glucan (CSBG and ASBG) were prepared by the NaClO-oxidation method using a procedure described previously^{11,12)}. Grifolan (GRN) was prepared by fermentation of the mycelium of *Grifola frondosa* as reported¹³⁾. The polysaccharide fraction (AgHWE) from *Agaricus brasiliensis* (= *A. blazei* Murill sensu Heinem) was prepared as described previously¹⁴⁾. Yeast mannan (Y-Man) was from Nakalai Tesque, Ltd. (Kyoto, Japan). Table 1 and Figure 1 show the characteristics of the β -glucan used in this study.

Table 2. List of serum samples

Species of animal	Remarks
Bovine	pooled serum; purchased from TRACE Scientific, Ltd.
Monkey, Goat, Rabbit, Dog, Guinea pig, Turkey	pooled serum; purchased from Japan Bio serum Co., Ltd.
Horse	total n=404; were bred at Azabu University (n=152) or eight breeding farms (n=252)
Chicken	n=166; were bred at Azabu University
Pig	n=158; were bred at Azabu University
Piglet	n=165; were bred at Azabu University

Serum samples

Bovine pooled sera were purchased from TRACE Scientific, Ltd. (Melbourne, Australia). Monkey, goat, rabbit, dog, guinea pig and turkey pooled sera were purchased from Japan Bio serum Co., Ltd. (Hiroshima, Japan). The sera obtained from healthy individuals tested negative for viruses in a microbiological analysis and normal in a serological analysis.

The horses (n=152), chickens (n=166), pigs (n=158) and piglets (n=165) were bred at Azabu University, and their sera which had been kept at -20°C were used. Twenty of these sera were pooled and used to determine the anti-BG antibody reactivity of each animal's serum. An aliquot of blood was collected from a vein at appropriate intervals using heparinized capillaries. After centrifugation, samples were stored at -20°C prior to the enzyme linked immunosorbent assay (ELISA) for anti-BG antibody (Table 2). Horse serum was also collected from eight breeding farms (A; n=37, B; n=58, C; n=12, D; n=20, E; n=25, F; n=32, G; n=17, H; n=51, total n=252) whose animals were healthy and showed no symptoms of infection. Table 2 shows the animal sera used in this study.

All owners enrolled in the study consented to procedures approved by the Clinical Research Review Committee of Azabu University.

ELISA for reactivity of anti-BG antibody

A 96-well Nunc plate was coated with the glucan preparation (CSBG, AgHWE, ASBG or GRN) or Y-Man in a 0.1M carbonate buffer (pH 9.6) by incubation at 4°C overnight. The plate was washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) and blocked with 1% gelatin (Wako Pure Chemical Co.) or bovine serum albumin (Sigma-Aldrich Co., MO, USA) PBST (GPBST or BPBST) at 37°C for 60 min. After being washed, the plate was incubated with serum at 37°C for 60 min. The sera were diluted in the 200 to 25,000 range, then washed with PBST and treated with peroxidase-conjugated anti-cow, pig, horse,

chicken, monkey, rabbit, goat, guinea pig, turkeys or dog IgG antibody (Sigma-Aldrich Co.) in GPBST or BPBST, and developed with a tetramethylbenzidine (TMB) substrate system (KPL Inc., MD, USA). Color development was stopped with 1M phosphoric acid and optical density was measured at 450 nm (reference wavelength: 630nm). An immune plate, Nunc 442404, F96 Maxisorp (Thermo Fisher Scientific Inc., Roskilde, Denmark) was used for all the ELISA experiments.

Competitive ELISA for specificity of anti-BG antibody

A 96-well Nunc plate was coated with CSBG in a 0.1M carbonate buffer (pH 9.6) by incubation at 4°C overnight. The plate was washed with PBST and blocked with 1% GPBST or BPBST at 37°C for 60 min. After being washed, serum was mixed with serially diluted polysaccharides (CSBG, AgHWE, ASBG, GRN or Y-Man) and then applied to the plate. The plate was incubated at 37°C for 60 min. It was washed with PBST and then treated with peroxidase-conjugated anti-cow, pig, horse, chicken, monkey, rabbit, goat, guinea pig, turkey or dog IgG antibody (Sigma-Aldrich Co.) in GPBST or BPBST, and developed with a TMB substrate system (KPL Inc., MD, USA).

Statistical analysis

The paired *t*-test was used to evaluate statistical significance for paired samples. $P < 0.05$ was considered significant in all analyses.

Results

Reactivity and specificity of anti-BG antibody in sera of livestock and domestic pets

First, to demonstrate the average reactivity of each animal's serum, we examined the reactivity of the anti-BG antibody in the pooled serum of cows, pigs, horses, chickens, monkeys, rabbits, goats, guinea pigs, turkeys or dogs by ELISA with various β -glucans as an antigen (Fig. 1, Table 1). Absorbance increased the concentration of these sera, and the response curve was different for each antigen (Fig. 2). The anti-BG antibody of each

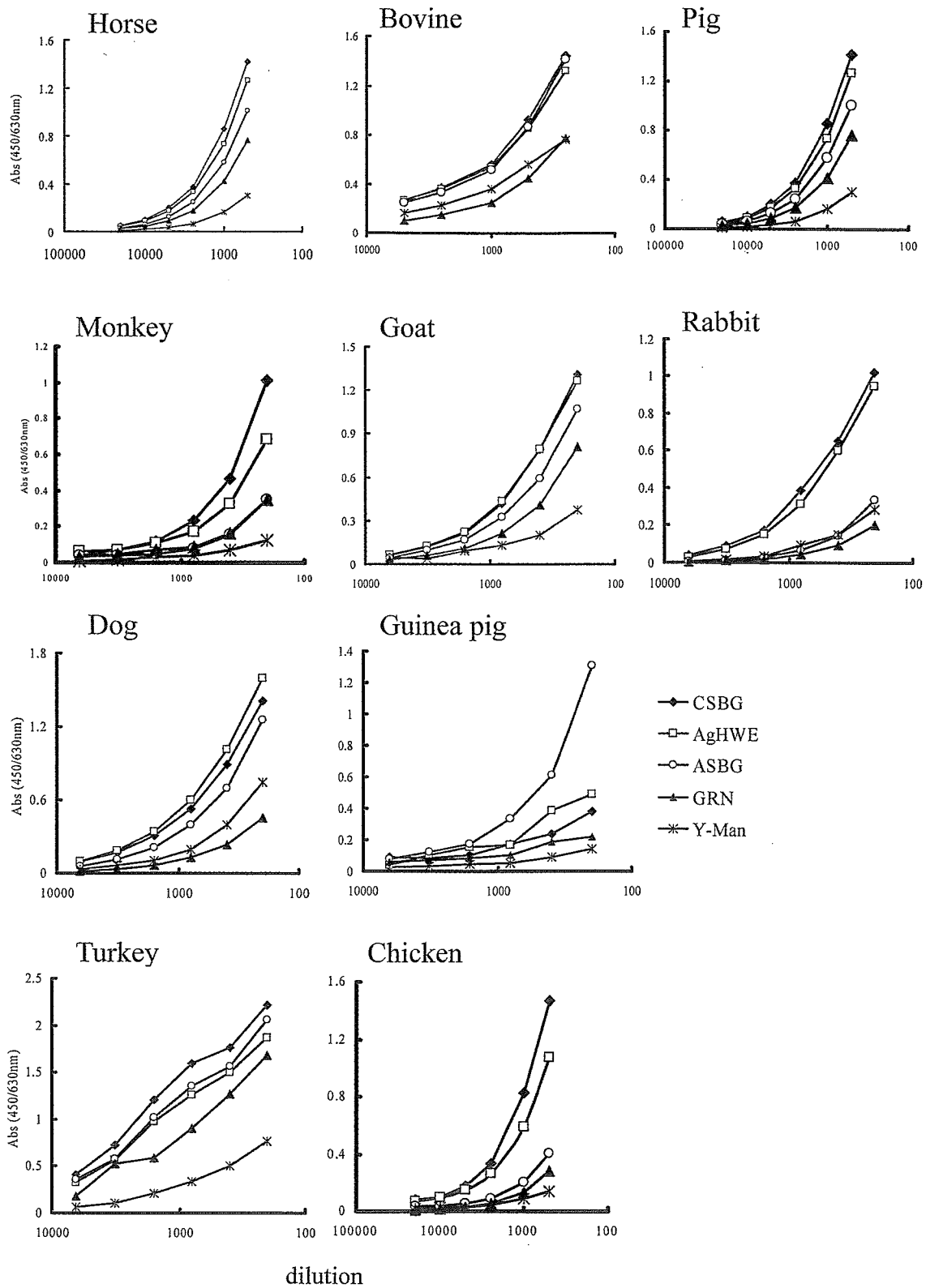


Fig. 2. Comparison of reactivity of animal sera to various polysaccharide-coated plates. An ELISA plate was coated with each polysaccharide (CSBG: \blacklozenge , AgHWE: \square , ASBG: \circ , GRN: \blacktriangle , Y-Man: $*$, 25 $\mu\text{g}/\text{ml}$). Sera were diluted and the amount of plate-bound immunoglobulin was determined with peroxidase-conjugated anti-cow, pig, horse, chicken, monkey, rabbit, goat, guinea pig, turkey or dog IgG antibody. Enzyme activity was measured by the addition of TMB substrate.

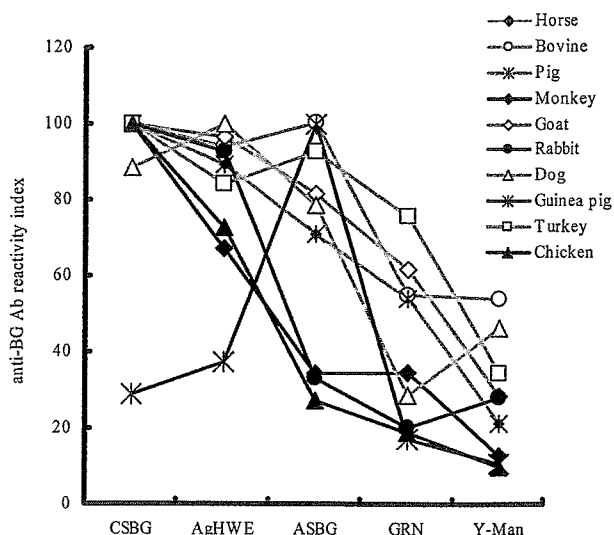


Fig. 3. Reactivity of animal sera to various polysaccharide-coated plates.

A reactivity index indicating the reactivity to the most reactive antigen at the lowest dilution as 100 in each animal in Fig. 2.

animal species was even detected in the thousand-fold dilution. Figure 3 gives a summary of the reactivity in each animal. In most animals, sera showed a high degree of reactivity to β -1,3-glucan containing a slightly branched long β -1,6-glucan segment, *Candida* solubilized cell wall β -glucan (CSBG), and a low level of reactivity to GRN, a 6-branched β -1,3-glucan. The reactivity to Y-Man used as a control antigen was also weak. Anti-BG antibody to AgHWE mainly composed of β -1,6-glucan or ASBG mainly composed of β -1,3-glucan was also detected. The rate of reactivity to ASBG and AgHWE was different among each species. Most animals showed a high degree of reactivity to AgHWE, especially the rabbits and chickens; on the other hand, guinea pigs showed a higher level of reactivity to ASBG. Anti-BG-antibody was present with a different reactivity in various animals.

We previously examined the cross-reactivity of the human anti-BG antibody by adding various glucans as a soluble competitive antigen to CSBG-coated plates. Human anti-CSBG antibody showed cross-reactivity to the anti-ASBG antibody and anti-AgHWE antibody. In this study, to additionally examine the characteristic of the anti-BG antibody in animal sera, epitopes cross-reactive to various glucans (Table 1, Fig. 1) were examined by competitive ELISA (Fig. 4). Strong inhibition was exhibited not by Y-Man but by CSBG, showing the antibody to be β -glucan specific. The reactivity was also inhibited by GRN, but to a lesser degree. A difference in the rate of inhibition was

observed among the animals. These results supported the results of the ELISA with various β -glucans.

Individual differences in the titer and reactivity of anti- β -glucan antibody

The reactivity and titer of the anti-BG antibody in individuals of animal species were examined in more detail. The titer of antibody to CSBG and GRN in the chickens was examined in individual animals (Fig. 5). The sera contained higher titers of anti-CSBG antibody than of GRN on average. However, a few individuals had higher titers of anti-GRN antibody than of CSBG. These results show individual differences in reactivity as well as titers in each species. The reactivity of the anti-BG antibody in horse serum was examined (Fig. 6), and showed greater reactivity to CSBG than to GRN.

In pigs, the anti-BG antibody titer was compared between the piglets and adults (Fig. 7). The average titer was higher for the pigs than piglets (pigs; 0.936, piglets; 0.650, *t*-test ($P < 0.05$)).

Next, to examine the influence of the breeding environment on the anti-BG antibody titer, we compared titers by breeding farm among horses. The anti-BG antibody titer in individuals of each farm differed (Fig. 8). However, the highest average absorbance among the clubs was 0.815, while the lowest was 0.494. The titer of the anti-BG antibody thus changed according to the breeding environment.

Discussion

We previously reported that an antibody to fungal cell wall β -glucan, anti-BG antibody, was present in human sera and played a role in host defense against pathogenic fungi⁹. In animals as well as humans, fungal infections often become a clinical problem, thus we examined the anti-BG antibody in animal sera. We first demonstrated the presence of the antibody to fungal cell wall β -glucan in each animal serum.

Host defenses against pathogenic fungi have been examined extensively using murine models. Immunity through T cells and neutrophils has been reported¹⁵, however, in pets and farm animals, it is little understood. In the present study, an antibody recognizing pathogenic fungal cell β -glucan was detected in livestock and domestic pets. We have demonstrated that the anti-BG antibody titer was significantly decreased in patients with deep mycosis (*aspergillus pneumonia* and *carinii pneumonitis*)¹⁶. Also, it was shown that the anti-BG antibody formed an antigen-antibody complex with fungal cell wall β -glucan and played a role in host defense against pathogenic fungi *in vitro*⁹. Torosantucci et al. showed that an anti-BG monoclonal antibody improved fungal burden following a systemic infection

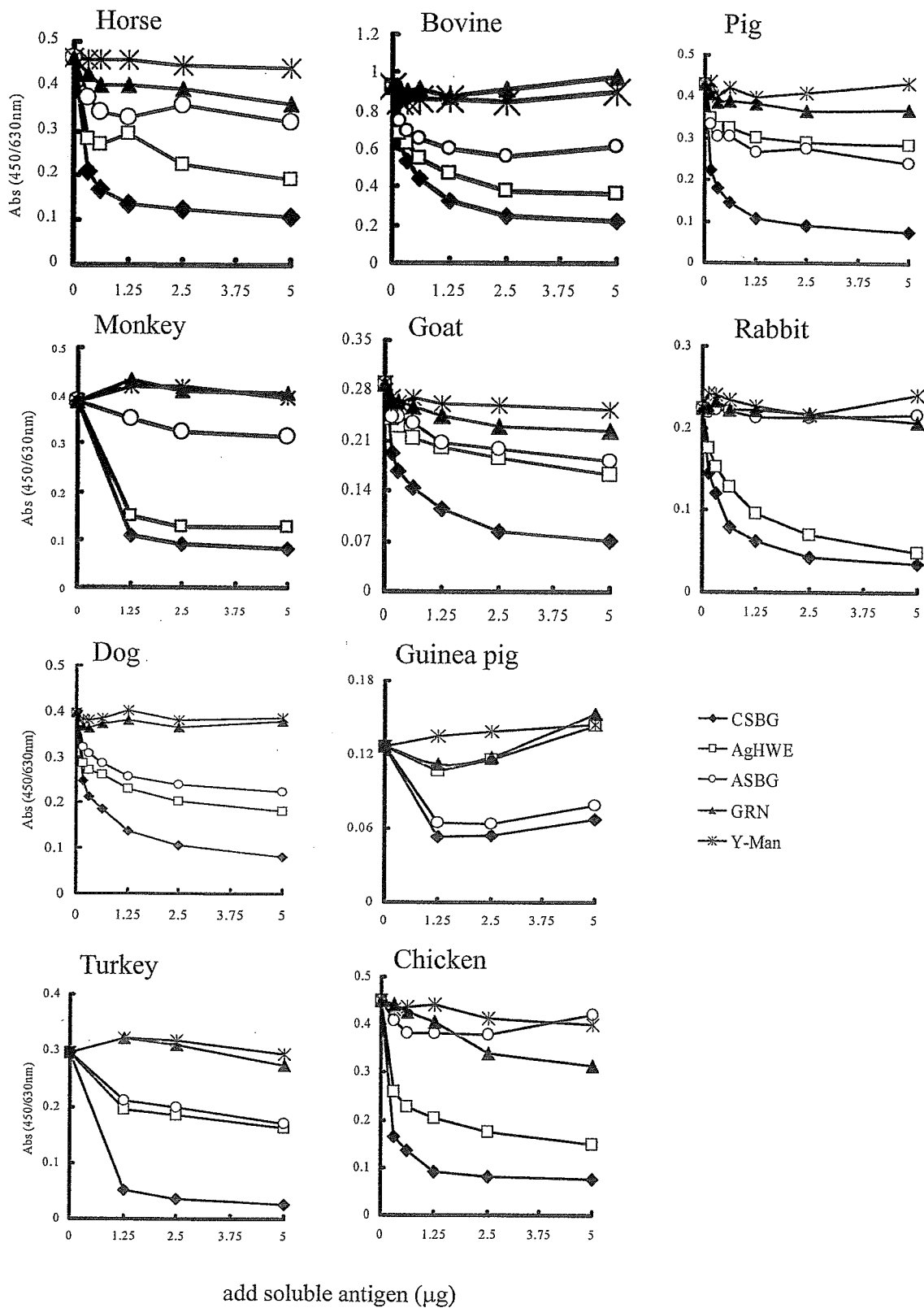


Fig. 4. Specificity of animal sera for plate-bound glucan.

An ELISA plate was coated with CSBG ($25 \mu\text{g/ml}$). Sera were mixed with serially diluted polysaccharides (CSBG: \blacklozenge , AgHWE: \square , ASBG: \circ , GRN: \blacktriangle , Y-Man: $*$) and then applied to the plate. The amount of plate-bound immunoglobulin was determined with peroxidase-conjugated anti-cow, pig, horse, chicken, monkey, rabbit, goat, guinea pig, turkey or dog IgG antibody. Enzyme activity was measured by the addition of TMB substrate.

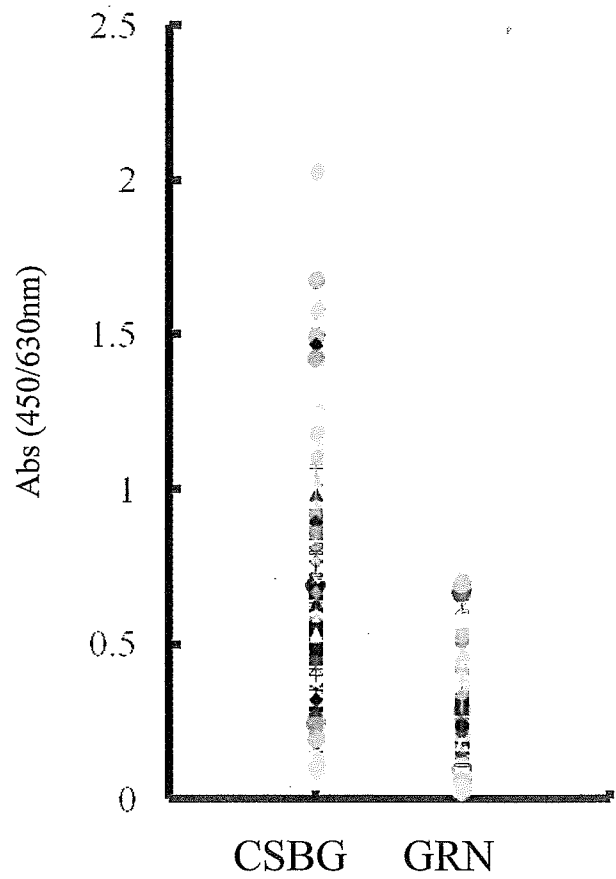
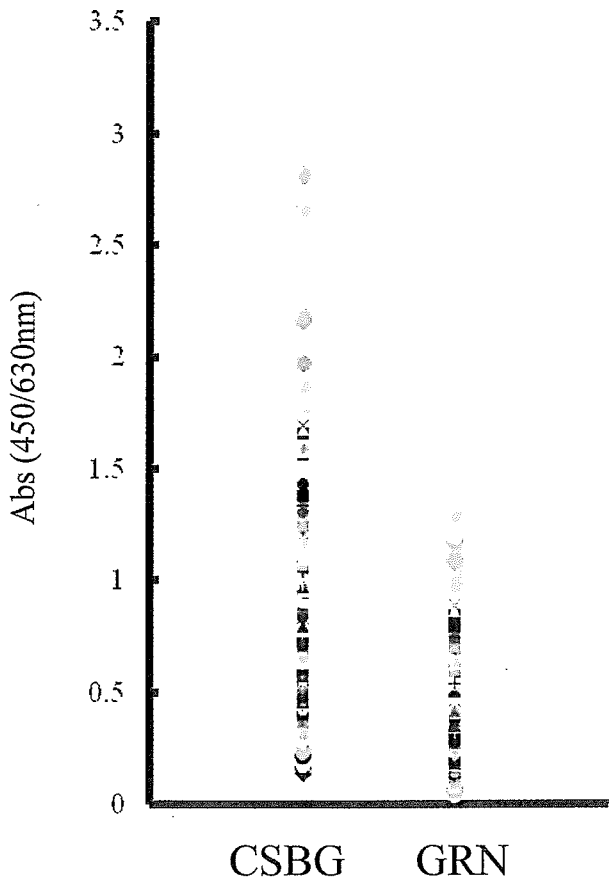


Fig. 5. Comparison of reactivity of chicken sera to CSBG or GRN.

An ELISA plate was coated with CSBG or GRN (25 $\mu\text{g}/\text{ml}$). Chicken sera (N=166) were diluted and the amount of plate-bound immunoglobulin was determined with peroxidase-conjugated anti-chicken IgG antibody. Enzyme activity was measured by the addition of TMB substrate.

Fig. 6. Comparison of reactivity of horse sera to CSBG or GRN.

An ELISA plate was coated with CSBG or GRN (25 $\mu\text{g}/\text{ml}$) and blocked before use. Horse sera (N=152) were diluted and the amount of plate-bound immunoglobulin was determined with peroxidase-conjugated anti-horse IgG antibody. Enzyme activity was measured by the addition of TMB substrate.

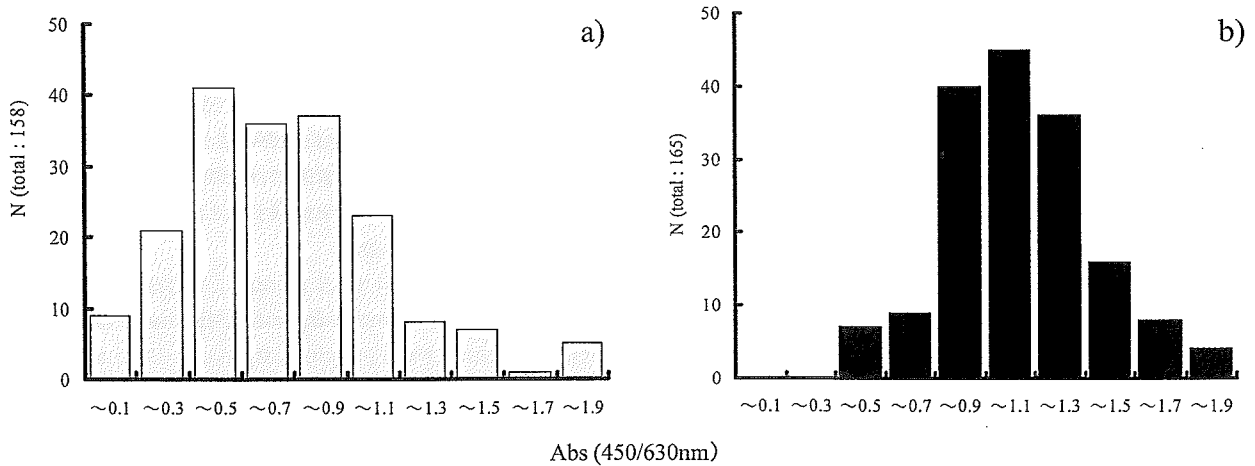


Fig. 7. Comparison of anti-BG antibody titers of piglet and pig sera.

An ELISA plate was coated with CSBG (25 $\mu\text{g}/\text{ml}$). The sera of a) piglets (N=158) and b) pigs (N=165) were added to the plate, and the amount of plate-bound immunoglobulin was determined with peroxidase-conjugated anti-swine IgG antibody. Enzyme activity was measured by the addition of TMB substrate.

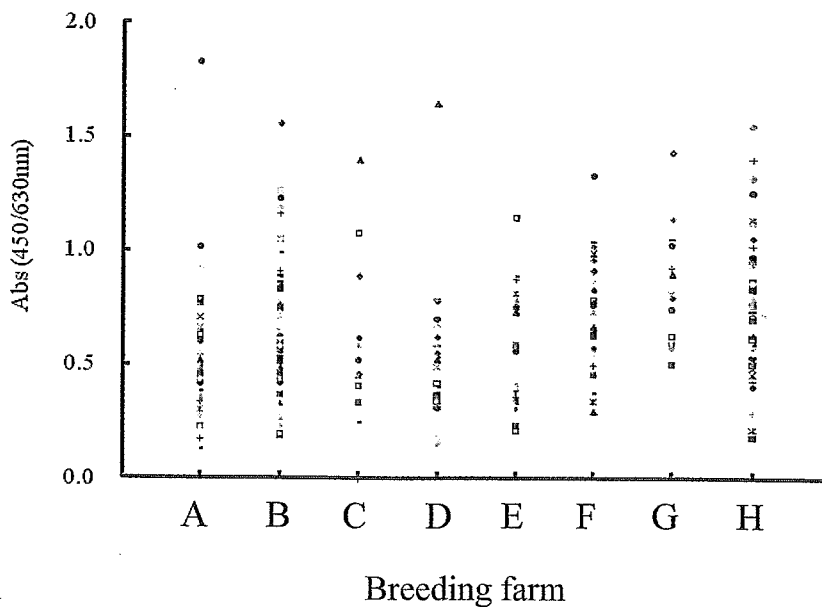


Fig. 8. Anti-BG antibody titers of horse sera from various breeding farms.

An ELISA plate was coated with CSBG (25 $\mu\text{g}/\text{ml}$). Horse sera from various breeding farms (A; n=37, B; n=58, C; n=12, D; n=20, E; n=25, F; n=32, G; n=17, H; n=51, total n=252) were added to the plate, and the amount of plate-bound immunoglobulin was determined with peroxidase-conjugated anti-horse IgG antibody. Enzyme activity was measured by the addition of TMB substrate.

with *C. albicans* and *A. fumigatus*¹⁷⁾. The anti-BG antibody in animal sera could have a similar protective role against fungal infection.

β -glucan is widely distributed in the natural world. We have prepared various β -glucans and analyzed their structure and properties, finding that the structure (the branching-ratio and length of the branched chain) varies with the source. In most animals, anti-BG antibody was highly reactive to CSBG: a *Candida* cell wall glucan composed of a slightly branched long β -1,6-glucan and β -1,3-glucan. On the other hand, it showed only a weak response to GRN: a 6-branched β -1,3-glucan from medium cultivated with the mycelial form of *Grifola frondosa*. Also, a comparison of the reactivity in chickens or horses revealed that individual differences existed. Most individuals showed high reactivity to CSBG, but some showed high reactivity to GRN. The reactive tendency in each individual was similar to the reactivity of pooled serum in chickens and horses; it would reflect the structure of the β -glucan exposed.

It was shown that the titer of the antibody to β -1,3-glucan or β -1,6-glucan was different in each animal. The chickens, rabbits and monkeys showed high titers of antibody to β -1,6-glucan, while the guinea pig showed high titer to β -1,3-glucan. It is known that fungi such as *Candida* spp. colonize the intestinal tract, and that the intestinal flora induces natural antibody

production¹⁸⁾. Zrimsek et al. also reported that a specific IgG antibody was generated in rabbits naturally infected with *Trichophyton mentagrophytes*¹⁹⁾. Hence, it was suggested that the anti-BG antibody was generated by fungi which colonized the mucosal surface, and the reactivity of this antibody in each animal species was reflected by its response to fungi.

We compared the anti-BG antibody titer between or within breeding farms for horses and found that a difference existed. This suggests that the anti-BG antibody titer was influenced by environmental factors such as the frequency of exposure to fungi and feed. Each breeding farm showed an individual difference. The finding that pig sera showed higher titers than piglet sera supported these results, suggesting that these differences were caused by the period of exposure and sensitivity to fungi.

In animals as well as humans, mycosis can cause problems such as mass infection. Because animals are often in contact with people, they can be the source of infection in human cases of mycosis²⁰⁾, thus making it important to control fungal infections in animals. For the diagnosis of fungal infections in animals, histological and cultural methods are the standard, but require time and labor. In humans, serodiagnoses such as the detection of cell wall antigen are applied. The anti-BG antibody titer in patients with fungal infections decreased, and fluctuated according to clinical conditions¹⁶⁾. Therefore,

anti-BG antibody is thought to be an index of the biological response and sensitivity to fungi.

In conclusion, this study showed that antibody to β -glucan, a major component of the fungal cell wall is present in the serum of livestock and domestic pets. This antibody is highly reactive to the cell wall β -glucan of pathogenic fungi. The anti-BG antibody is believed to interact with fungal cell wall β -glucan and to participate in the immune-response to pathogenic fungi.

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