Article

A Study on the Glycan Specificity of Natural Antibody Repertoires in Rodents

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Inbred strains of mice and rats are widely used in preclinical investigations evaluating the effectiveness of glycan-based biomecines, however, the glycan specificity repertoires of serum Abs in rodents have not been fully characterized. In the present study, serum antibodies in naïve mice and rats of different inbred strains were analyzed for specificity against 4 representative carbohydrate structures including PGA (1,4-linked α-D-galactopyranosyluronic acids), β-glucan, mannan and α-glucan (dextran). Mannan was not recognized by serum Abs from any of the mouse and rat strains. Serum IgM in naïve F344, BN and Lewis rats recognized PGA and β-glucan and, less strongly, dextran. High titer circulating IgM against PGA were found in mice of BALB/c, C57BL/6, C3H/NeH and BXSB strains. C3H/NeH was the only strain which also produced low titer IgM against β-glucan and dextran. Age-related production of high titer IgM, IgA and IgG Abs against β-glucan was observed in BXSB mice. Intraperitoneal immunization of BALB/c and C57BL/6 mice with β-glucan elicited strong IgM responses, while immunization with PGA also led to an increase of anti-PGA IgM Ab titers. These results provide useful information on the characteristics of glycan-specific natural antibody repertoires in rodents. *Cellular & Molecular Immunology*. 2009;6(6):453-459.

Key Words: mouse, autoantibody, glucan, autoimmune disease

Introduction

Glycans, displayed on the surface of bacteria, fungi and parasites or on self-tissue cells, represent a major class of antigens recognized by the immune system, particularly by antibodies (Abs) produced by B lymphocytes and pattern recognition receptors of the innate immune cells (1-12). Malignant cells have also been found to express unique glycan moieties (i.e. tumor-associated carbohydtrate antigens, TACAs) that are useful biomarkers for cancer diagnose and immunotherapy (13-16). Vaccines conjugating TACAs or pathogen-derived carbohydrate antigens with carrier proteins have been developed to induce glycotope-specific humoral responses *in vivo* (13, 16, 17). Various polysaccharide preparations (e.g. β-glucans) extracted from medicinal herbs have been widely used as immunopotentiating medicines for treatment of cancers or infectious disorders (18-23). Preclinical studies evaluating the effectiveness of glycan-based medicines or vaccines rely on experimental animals, particularly inbred strains of mice and rats (7, 11, 12, 23). In the last two decades, glycan-specific natural Ab repertoires in humans have been intensely studied with much progress (1-6). However, glycan specificity of the rodent natural Ab repertoires, which may substantially influence the outcome of preclinical studies on glycan-based vaccines or drugs, has not yet been fully characterized. The present study was undertaken to analyze mouse and rat serum Ab repertoires against polysaccharides representing 4 major glycan structures including PGA (homono-polysaccharides of 1,4-linked α-D-galactopyranosyluronic acids), α1,6-glucan (dextran), α1,3-mannan and β-glucan. Results arising from this study should provide useful clues on the glycan specificity of circulating Abs in rodents.

Materials and Methods

Mice and rats

BALB/c, C57BL/6 and C3H/HeN mice of 2 or 6 months of

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Abbreviations: PPS, polysaccharides extracted from the roots of *Polyporus;* APS, polysaccharides extracted from the roots of *Astragalus membranaceus.*

Table 1. Structural characteristics of the polysaccharides utilized in this study

Groups	Origin	Structural characteristics
β-glucan	PPS, extracted from *Polyporus umbellatus Fries* (*Zhuling*)	β-glucan composed of 1,6-linked backbone and 1,3/1,4-linked branches
α-glucan	Dextran, synthetic	α-1,6-linked unbranched glucan
PGA	APS extracted from *Astragalus membraneous* (*Huangqi*)	α-1,4-linked-D-galactopyranosyluronic acids with some methyl esterifed carboxyl groups as backbone
	PGA extracted from plant cell walls	α-1,4-linked -D-galactopyranosyluronic acids
Mannan	Mannan, extracted from *Saccharomyces cerevisiae*	α-1,3-linked mannan

age were purchased from the Experimental Animal Division of Peking University Health Sciences Center, Beijing, China. BXSB (H-2^b) mice, originally imported from Jackson Laboratories, USA, were bred and maintained at this department. Female F344, Lewis and BN rats of 3 months of age were provided by Unilihua Bioscience Center, Beijing, China. All animal experiments were carried out, with the permission of Beijing Experimental Animal Management Authority, Beijing, China, at the animal facilities of this department.

Polysaccharides employed in this study

Synthetic dextran (MW 70,0000), an α-1,6-glucan, and *Saccharomyces cerevisiae* mannan (α-1,3-linked mannosides, M7504) were purchased from Sigma (USA). PPS is polysaccharides extracted from the roots of *Polyporus umbellatus (Pers) Fries* (*Zhuling*, a popular medicinal herb in China). APS is polysaccharides extracted from medicinal herb *Astragalus membraneous* (*Huangqi*). *Zhuling* and *Huangqi* slices were extracted 3 times with boiling water. The supernatant was applied to a DEAE-Sephacel (2.6 × 100 cm) column and the bound material eluted with a linear gradient of NaCl (0-2 mol/L NaCl). Carbohydrate concentration in the fractions was determined using the phenol sulfuric acid method. The fractions thus obtained were pooled and precipitated 3 times with ethanol. The resultant poly-saccharide extract was dialyzed against several changes of water and then lyophilized. Carbohydrate content of the final product was > 85% with trace protein and nucleic acid contamination. The molecular weight of the extract was approximately 1.96×10^6 as determined by gel filtration. Analysis by TLC and gas chromatography showed that the polysaccharide was 90% glucose, with mannan, galactose and xylose also present.

Immunization and blood sampling

For blood sampling, mice and rats were bled from the tail vein and the blood samples were centrifuged at 10,000 ×g for 10 min, and the sera were collected and stored at -20°C. For polysaccharide immunization experiments, BALB/c mice (5 per group) were immunized intraperitoneally with 200μg PPS or APS in PBS. The mice were bled every week after the immunization for up to 8 weeks.

Polysaccharides-based ELISA

Specific Abs to glycans were measured by ELISAs as previously described (24). Briefly, flat bottom 96-well microtitre plates (Corning-Costar) were coated with 50 μg/ml polysaccharides in 0.1 M carbonate-bicarbonate buffered saline (pH 9.6) at 4°C overnight. The plates were washed with 0.05% Tween 20 (Sigma, St. Louis, Mo.) in PBS three times between each stage. Each plate was blocked with 10% FCS in PBS for 2 h at 37°C. Serum samples were diluted 1:200, or as indicated, in 2% FCS in PBS and incubated in the ELISA wells for 2 h at 37°C. Detection of IgM, IgG or IgA was done using goat anti-mouse IgM, IgG or IgA coupled to Horseradish peroxidase (Southern Biotechnology Associates Inc., Birmingham, AL.) diluted 1:4000 in PBS-Tween and incubated for 1h at 37°C. The reaction was developed with 100 μl of O-phenylenediamine (OPD, Sigma) for 5 min and stopped with 100 μl 3M H_2SO_4. Optical density (OD) was measured at 492 nm in an ELISA spectro photometer (Flow Laboratories, Irvine, UK).

Statistical analysis

All experiments were repeated at least 3 times and the results are expressed as mean±standard error of the mean (SEM). Comparison of the data was performed using the Student's *t* test. Significance was defined as a p value of $< 0.05\%$. Statistical analysis was performed using SPSS software.

Results

Glycan specificity of circulating Abs in different strains of mice

A panel of polysaccharide preparations, summarized in Table 1, was employed as coating Ags in ELISAs which allowed us to characterize serum Abs specific for PGA, dextran, β-glucan (PPS) and α1,3-mannan. As illustrated in Figures 1A and 1B, serum IgM of 2-month-old female C57BL/6 (H-2^b) and BALB/c (H-2^d) mice specifically recognized PGA but not dextran, PPS or α1,3-mannan. Analysis of sera from 2-month-old BXSB (H-2^b) mice produced similar results (see below). C3H/HeN (H-2^k) mice differed from the other 3 strains in that they produced not only high titer IgM specific for PGA but also relatively low titer Abs against β-glucan

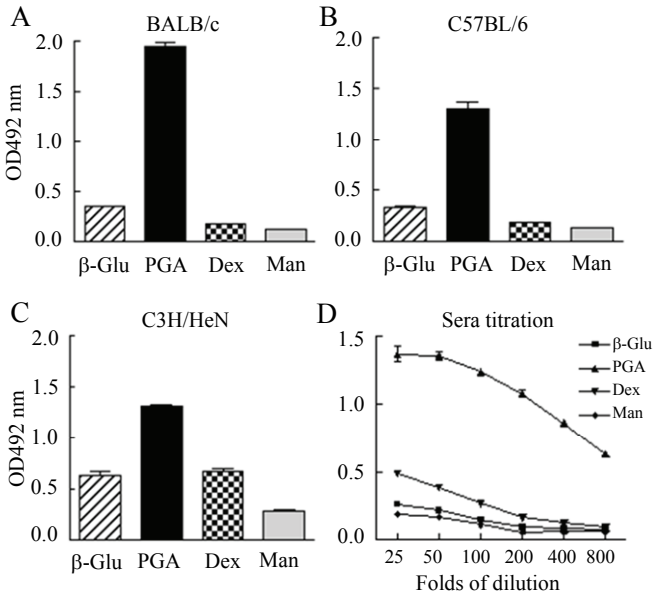

Figure 1. Glycan-specific natural Abs in mice. Serum samples (equal portion mixture from 5 mice per group, 1:200 diluted) from 2-month-old female BALB/c (A), C57BL/6 (B), and C3H/HeN (C) mice were assayed in ELISAs using polyvinyl plates coated with PPS (β-Glu), PGA, dextran (Dex) or mannan (Man). The detection Ab was HRP-conjugated goat-anti-mouse IgM with OPD as substrate and the results expressed as absorbance at wavelength 492 nm (OD492 nm) with standard deviation. In Panel D, a serum sample from BALB/c mice were serially diluted and assayed for IgM in ELISAs based on PGA, PPS, dextran or mannan.

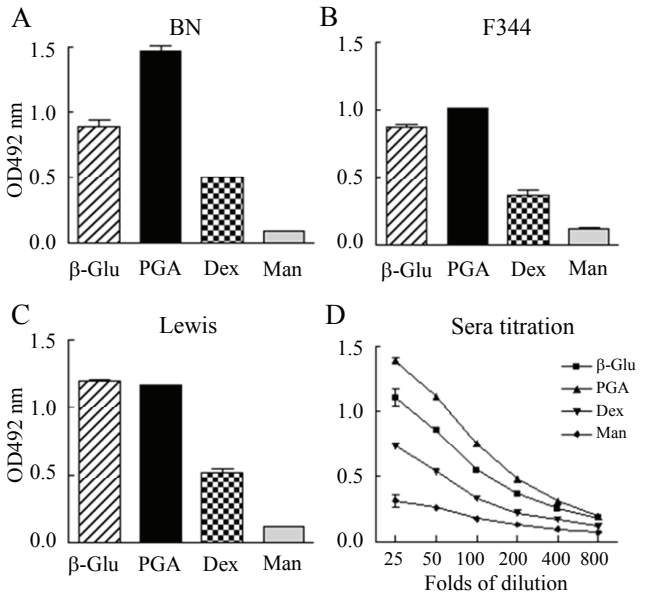

Figure 3. Glycan-specific natural Abs in rats. Serum samples (1:200 diluted equal portion mixture from 5 rats per group) from 3-month-old rats of BN, F344 and Lewis strains were assayed in ELISAs using polyvinyl plates coated with PPS (β-Glu), PGA, dextran (Dex) or mannan (Man). Detection Ab was HRP-conjugated goat-anti-rat IgM with OPD as substrate and the results expressed as OD492 nm with standard deviation. In panel D, mixed sera from adult F344 rats were serially diluted and assayed in ELISAs based on PGA, PPS, dextran or mannan.

and dextran (Figure 1C). The titer of PGA-specific IgM in normal mouse serum (NMS) was impressively high, as they were detected in serially diluted serum sample from naïve mice with an end point of 1/1600 (Figure 1D). When NMS was pre-incubated with APS (PGA-containing polysaccharides extracted from medicinal herb *Astragalus membraneous*), or PPS, or dextran, or mannan, only APS was able to abolish its ability to bind PGA in ELISAs (Figure 2), confirming the specificity of the ELISA systems employed in this study. No IgG or IgA capable of binding any of these polysaccharides were detected in parallel experiments (data not shown).

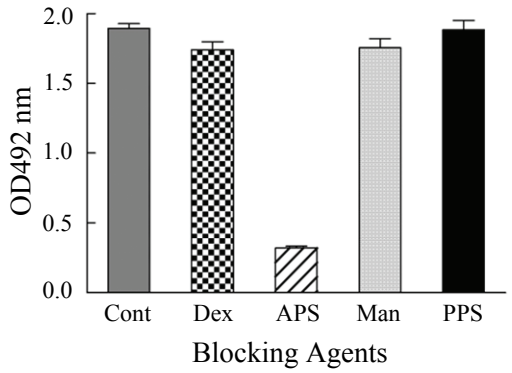

Figure 2. Competition assays. Serum samples (1:200 diluted) from 2-month-old female BALB/c mice were incubated at room temperature for 1 h in the presence, or absence (Cont), of PPS, or APS, or dextran (Dex), or mannan (Man) at 1 mg/ml and then assayed in PGA-based ELISAs. The detection Ab was HRP-conjugated goat-anti-mouse IgM and the results expressed as OD492 nm with standard deviation.

Glycan specificity of circulating Abs in different strains of rats

Serum samples from 3-month-old female F344, Lewis and BN rats were also assayed for anti-glycan Abs using polysaccharides-based ELISAs. As illustrated in Figure 3, serum IgM from all 3 strains of rats specifically recognized PGA, PPS and, less strongly, dextran, but not α1,3-mannan. Titration of Lewis rat sera in ELISAs confirmed this observation and showed that the titer of rat anti-PGA IgM Abs was approximately one fifth of that in mice (Figure 3D). No IgG or IgA Abs specific for any of these glycans were detected in rat sera in parallel experiments (data not shown).

Anti-β-glucan serum Abs in aged BXSB mice

It has been shown that natural Abs have substantial influence on the development of autoimmune diseases (25). BXSB mice represent one of the most extensively studied animal models for human systemic lupus erythematosus (SLE), a

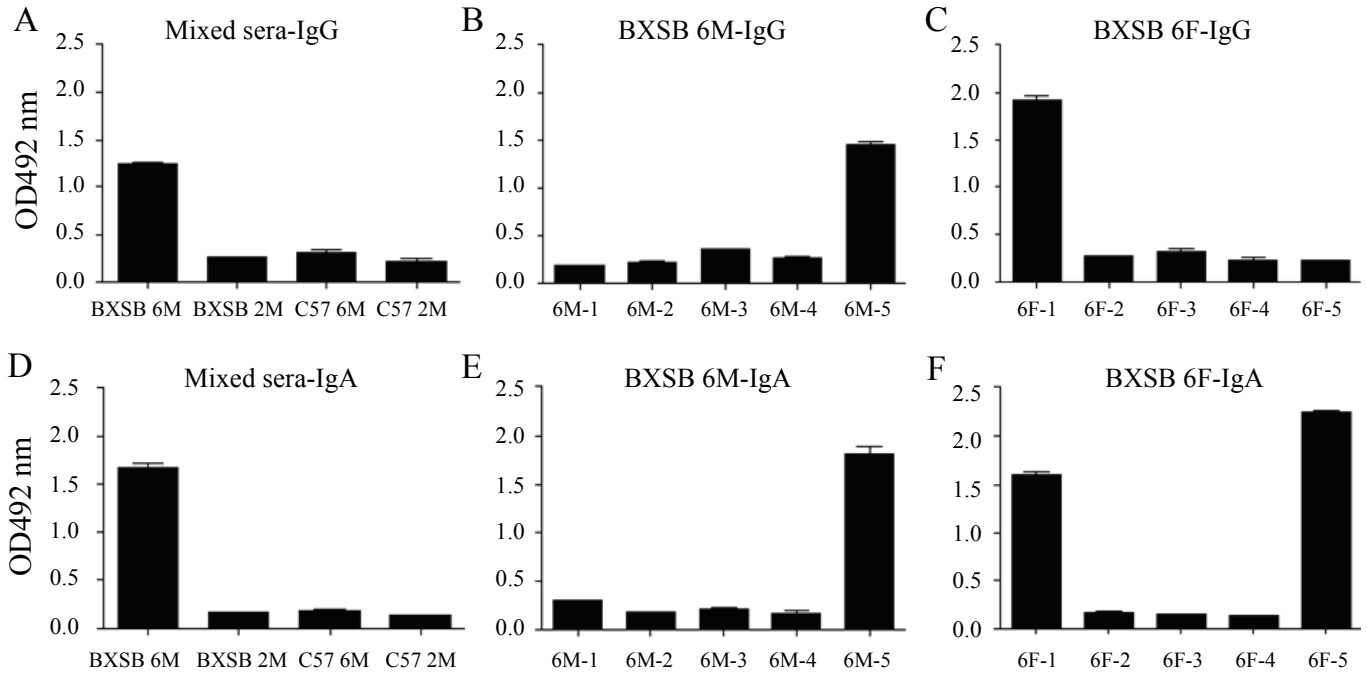

Figure 4. Anti-β-glucan Abs in BXSB mice. Serum samples (1/200 diluted, equal portion mixture from 5 mice per group) from male BXSB mice of 2 months (BXSB 2M), or 6 months (BXSB 6M), of age were compared for anti-β-glucan IgG (A) and IgA (D) Abs in PPS-based ELISAs. Sera from male C57BL/6 mice of 2-months (C57 2M), or 6-months (C57 6M), of age were included as controls. Individual serum samples from 6-months-old male (6M-1 to 6M-5) and female (6F-1 to 6F-5) BXSB mice were assayed in PPS-based ELISAs for IgG (B and C) and IgA (E and F) Abs. The detection Abs were HRP-conjugated goat-anti-mouse IgG (A, B, C) or IgA (D, E, F) with OPD as substrate. OD 492 nm of each well was measured and the results are shown as mean with standard deviation bars.

systemic autoimmune disorder characterized by the production of autoantibodies against a variety of autoantigens, particularly dsDNA and nuclear proteins (26-28). It was therefore of interest to investigate if the glycan-specificity of BXSB natural Abs differs from that of the other strains of mice. Compared with that from BALB/c and C57/BL6 mice, serum samples of 2-months-old BXSB mice showed a similar pattern of recognition towards glycan preparations employed in this study (Figure 4). However, β-glucan-specific IgM as well as IgG and IgA Abs were detected in mixed sera from 6-month-old BXSB, but not C57BL/6, mice (Figures 4A and 4B). When serum samples from the 6-month-old BXSB mice were individually assayed for anti-β-glucan Abs, much individual variation was observed. As shown in Figures 4C and 4D, 2 out of 10 BXSB mice were high producers of β-glucan-specific IgG, while 3 out of the 10 were high producers of anti-β-glucan IgA. It should also be noted that the prevalence of anti-β-glucan IgG and IgA Abs in aged BXSB mice was unrelated to sex, thereby excluding a possibility for direct association with murine lupus which mostly occurs in BXSB males rather than females (26-28).

Murine B cell response to stimulation with APS and PPS in vitro and in vivo

As shown in Figure 5, BALB/c and C57BL/6 mouse splenic B cells proliferated in response to stimulation with APS and PPS *in vitro*. The responding B cells also produced IgM Abs against PGA and β-glucan, respectively (data not shown), excluding the possibility that clonal deletion of β-glucan-specific B cell clones was responsible for the lack of β-glucan-specific natural Abs in these animals. We also wondered whether the circulating natural Abs (e.g. anti-PGA IgM) would influence (inhibit) humoral responses against relevant carbohydrate immunogens (e.g. APS), which is of particular concern for the design of vaccines aimed at inducing glycan-specific humoral immunity *in vivo*. In the experiment shown in Figure 6, BALB/c mice were i.p. immunized with APS, dextran, mannan or PPS, and then monitored for specific IgM Abs in their sera at different time points thereafter. Production of specific IgM was detectable 3 days after immunization with APS or PPS, reaching a plateau by day 7 (Figures 6A and 6B). Titration of the day 7 sera indicated that the titer of serum IgM against β-glucan increased approximately 10 folds in mice receiving PPS immunization, while an approximately 6 folds increase in the titer of serum IgM against PGA was observed in APS-immunized animals (Figures 6C and 6D).

Discussion

Our results indicate that IgM reactivity repertoires against glycan antigens in rodents are practically homogeneous within inbred strains and largely conserved in the species.

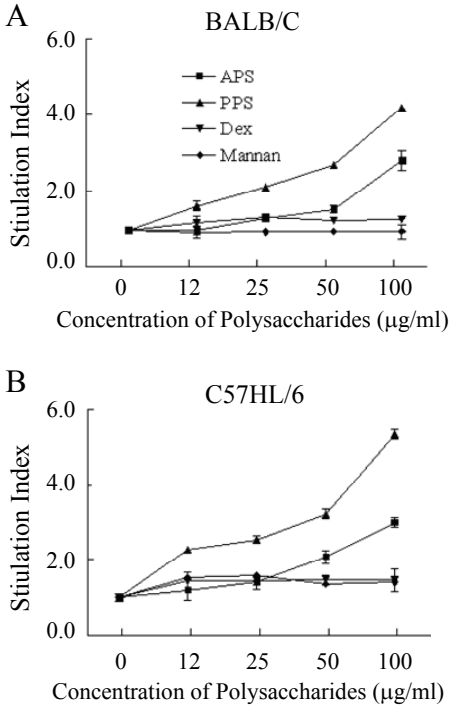

Figure 5. Proliferation assays. Freshly prepared BALB/c and C57BL/6 mouse splenocytes were stimulated with PPS, dextran (Dex), APS or mannan in a standard 72h proliferation assay. ^{3}H-TdR was added to the cultures for the last 8 h of incubation and then the ^{3}H-TdR incorporation (CPM) of each well counted. The results are expressed stimulation index calculated using medium only well as reference.

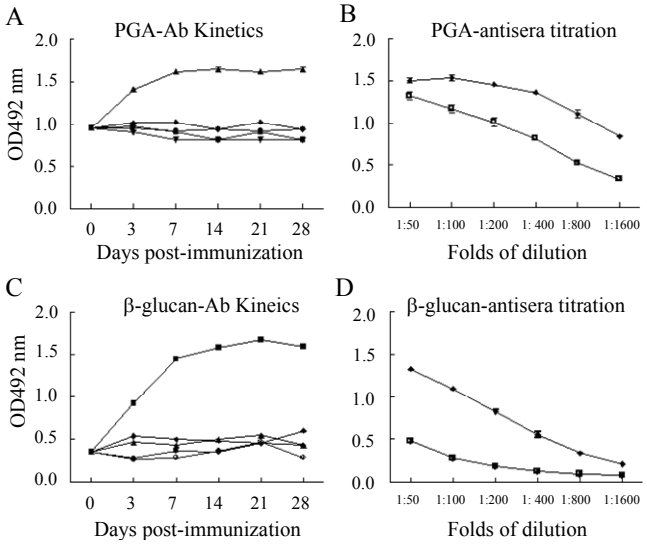

Figure 6. IgM responses induced by glycan immunization in mice. BALB/c mice (5 per group) were intraperitoneally injected with APS (upward triangle), or PPS (square), or dextran (diamond), or mannan (downward triangle) in PBS (200μg/100μl/ mouse), or PBS alone (circle) as control. The mice were bled on days 3, 7, 14, 21 and 28 and the sera assayed for IgM against PGA (A) or PPS (B) in ELISAs. The day 7 sera from mice immunized with APS (upward triangle) or PPS (downward triangle) were also serially diluted and compared with NMS (square) for IgM against PGA (C) or PPS (D). The detection antibody was HRP-conjugated goat-anti-mouse IgM with OPD as substrate and the results expressed as OD492nm with standard deviation.

For instance, the natural Abs of all 3 strains of rats (F344, Lewis and BN) strongly recognized β-glucan and PGA, and less strongly dextran (Figure 3). High titer circulating IgM against PGA were found in naïve mice of BALB/c, C57BL/6, C3H/NeH and BXSB strains (Figures 1, 3). C3H/NeH was the only mouse strain producing relatively low titer IgM Abs against β-glucan and α1,6-glucan (Figure 1C), reflecting genetic imprint (influence) in shaping the natural Ab repertoires of mice. It should be noted that previous studies also documented the influence of immune-related genes (e.g. MHC) on repertoire specificity of circulating Abs (29, 30). The use of congenic mice should allow more detailed analysis on genetic factors controlling the anti-glycan natural Ab repertoires in rodents.

There seems to be shared glycotope specificity by natural Ab repertoires across species. For example, PGA is not only a potent carbohydrate Ag for natural Abs in mice and rats, it is also recognized by serum IgM in human neonates during the first few weeks after birth, although anti-PGA Abs become undetectable in most healthy human adults (H. Dai, unpublished observation). Anti-α1,3-mannan natural Abs were hardly found in mice and rats (Figures 1, 3), which is similar to our previous finding that anti-mannan serum Abs (ASCMA) were absent in the sera from the majority of healthy human subjects. Interestingly significantly higher prevalence of anti-mannan Abs was found in patients with various autoimmune diseases (24).

It is generally accepted that the pecificity of natural Ab repertoires is shaped by not only genetic factors but also environmental stimuli. However, whether production of natural Abs against glycans relies on selective stimulation and activation of B, or B1, cells by environmental antigens remains a matter of debate. Bos and colleagues showed that natural Abs against peptidoglycan polysaccharide complexes (carbohydrate antigens of bacterial origin) were found in the serum of BALB/c mice that had been maintained in conventional SPF facilities, but were severely reduced in mice raised in a germ-free environment and fed a chemically defined, ultra-filtered diet, suggesting a positive role for exogenous antigen stimulation in shaping the natural Ab repertoires against glycans (8). However, the clear difference in IgM specificity towards β-glucan between mice and rats (both maintained in conventional SPF facilities) argues against this notion (Figures 1, 3). Haury et al also reported that the repertoire of serum IgM in mice is largely independent of external antigenic contact (31).

β-glucan, known as a pathogen-associated molecular pattern (9, 10), is found in the cell walls of various pathogenic or non-pathogenic microorganisms such as fungi. High titer IgM, IgG and IgA Abs against PPS have been found in sera from virtually all healthy human adults,

presumably as a result of adaptive immune response induced by microorganisms carrying the β-glucan moieties (manuscript in preparation). The substantial difference between natural Ab repertoires in humans and mice raises an important concern for preclinical studies using mouse models on β-glucan-based drugs or vaccines. Mouse strains lacking β-glucan-specific natural Abs (e.g. C57BL/6 and BALB/c) may be very responsive to treatment with β-glucan-based medicines, while the pre-existing serum Abs against β-glucan in humans may interfere with the biological activities of β-glucan *in vivo*.

Another important finding of this study is that high titer serum Abs, including IgM, IgG and IgA classes, were found in 6-months-old BXSB mice (Figure 4). Given that these animals had been maintained in SPF facilities, production of the anti-β-glucan IgG and IgA Abs is likely a result of adaptive immune response against autoantigens. It should also be noted, however, the prevalence of anti-β-glucan Abs in these animals was unrelated to sex, thereby excluding a dominant role for such Abs in immunopathogenesis of the disease.

Taken together, characterization of serum Ab glycan specificity in rodents will help us better understand the nature of natural Ab repertoires and also their formation control mechanisms. It also has important implications for the design and interpretation of preclinical studies using rodent models on carbohydrate-based medicines or vaccines. Further studies are merited for a more complete picture of the glycan specificity repertoires of natural Abs in rodents and also humans.

Aknowledgements

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