Boosted Rat Natural Xenoantibodies Cross-React with *Enterococcus faecalis* by Targeting Melibiose and L-Rhamnose

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Key Words

Xenoantibody · Natural antibody · *Enterococcus faecalis* · Melibiose · L-Rhamnose

Abstract

Natural antibodies include a subset described as xenoantibodies considered to be directed at microorganisms and also cross-react with antigens of unrelated species. In this study, we generated T-cell-independent (TI) and T-cell-dependent (TD) xenoantibodies in Lewis rats with hamster and pig blood injections. TI anti-hamster and anti-pig IgM and IgG xenoantibodies cross-reacted with *Enterococcus faecalis* but not with *Escherichia coli* isolated from the blood of Lewis rats after cecal ligation and puncture (CLP). TI anti-pig IgM xenoantibodies also showed some reactivity with two human blood isolates of *E. faecalis*. In contrast, TD xenoantibodies did not show any reactivity with rat or human bacteria. TI and TD anti-hamster and anti-pig IgM and IgG xenoantibodies showed cross-reactivity with lymphocytes and endothelial cells from species distinct to that used for immunization. Glycan array analysis and inhibition assays identified antibodies against melibiose and L-rhamnose as mediators of anti-hamster and anti-porcine xenoantibody cross-reactivity with *E. faecalis*. A rise in TI anti-hamster and anti-pig

xenoantibodies was accompanied by decreased survival of Lewis rats in a low-severity sepsis model of CLP. Therefore, TI xenoantibodies in the rat include anti-carbohydrate antibodies reactive to bacteria of endogenous flora. Enhancement of these antibodies may result in more severe infectious diseases caused by these microorganisms.

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Introduction

All individuals have circulating IgM and IgG natural antibodies that react with antigens in the absence of any evidence of prior exposure to them. As a general characteristic, these antibodies are polyreactive, exhibit modest antigen-binding affinity, are directed against carbohydrate antigens, and are due to the direct stimulation of antibody production by T-cell-independent (TI) pathways [1]. The generation of natural antibodies is considered an innate and primitive humoral immune response, which prevents autoimmunity and provides a rapid, but not specific, protection against commensal organisms and low inocula of pathogens [2].

Natural antibodies include a subset that react with antigens expressed on cells and tissues of unrelated species

described as xenoantibodies [3]. These antibodies are considered to be directed at microorganisms that cross-react with structurally similar xenoantigens. Thus, in animals, in the absence of environmental antigenic stimulation by microbes and food antigens, xenoantibodies do not develop until the protection is removed and normal bowel and environmental flora is established [4]. In humans, xenoantibodies comprise IgM and IgG directed against the galactose- α 1,3-galactose (α Gal) carbohydrate epitope [5], which is expressed in most mammalian species. Anti- α Gal antibodies react with various bacteria, including strains of *Escherichia coli*, *Klebsiella* and *Salmonella* [6, 7], and drop after antibiotic treatment that removes Gram-negative enteric flora [8]. These antibodies also bind to senescent human erythrocytes and tumor cells [9]. As occurs with natural antibodies, the xenoantibody response mediated by anti- α Gal antibodies in humans and in α Gal knockout mice, which lack the α Gal epitope and produce anti- α Gal antibodies like humans, initially involves the use of a restricted population of Ig germ-line genes before any rearrangement [10, 11].

The hamster-to-rat xenotransplantation model provided evidence for an early xenoantibody response characterized for the involvement of IgM TI antibodies that peaks at approximately 7 days and returns to baseline levels after 21 days [12]. Serum passive transfer experiments showed that IgM fractions from day 4, but not from days 21–40, caused hyperacute rejection of hamster xenografts. Genetic analysis demonstrated that the genes encoding these antibodies were used in the original germ-line configuration, such as natural TI antibodies, intended to react with infectious agents [12]. Hamster-to-rat xenotransplantation also induces IgG antibodies from all isotypes, which peak at 21–28 days after xenotransplantation, as well as causing hyperacute rejection of hamster xenografts in serum passive experiments [12]. The predominance of IgG antibodies at day 20 is associated with somatic mutations in the maturation of these antibodies, indicating that a T-cell-dependent (TD) pathway is involved in xenoantibody production at this time. Rat exposure to distantly related species such as pig is also associated with the generation of TI anti-pig xenoantibodies in germ-line configuration [13]. However, the relationship between rat natural xenoantibodies and antibodies to microorganisms is hypothetical since there is no evidence of germs recognized by rat xenoantibodies so far. To gain insight into this humoral immune response, we boosted TI (natural) and TD (adaptive) xenoantibodies in Lewis rats and investigated whether these antibodies bind to bacterial antigens and modify the response to infections.

Material and Methods

Animals

Lewis rats (weighing 200–250 g) and Golden Syrian hamsters (weighing 100–150 g) were purchased from Interfauna Harlan Iberica SL (Barcelona, Spain). Animals were maintained at the University of Barcelona (Bellvitge Campus) animal facility under controlled conditions of temperature (20–22°C) and humidity, with 12-hour light/12-hour dark cycles, and with food and water given ad libitum. Animals were anesthetized by isoflurane inhalation: deep anesthesia for hamsters (cardiac puncture), middle anesthesia for rat blood draw and light anesthesia for rat injections. All animal procedures were supervised and approved by the local ethics committee for animal experimentation and by the Catalan Government.

Rat Immunization

Two protocols of hamster or pig blood injections were used in rats in order to produce a pattern of predominantly TI or TD xenoantibodies. For TI, 3 intraperitoneal injections of 1 ml xenogeneic blood (every other day, on days 0, 2 and 4) were given, and blood was drawn on days 0 (before injection), 5, 8 and 20. For TD, 3 intraperitoneal injections of 1 ml xenogeneic blood (every other week, on days 0, 14 and 28) were administered, and blood was extracted on days 0 (before injection), 28, 40 and 55. Control animals (C-TI and C-TD) were subjected to 3 intraperitoneal injections of phosphate-buffered saline (PBS), and blood was collected on the same days as for TI and TD xenoantibody generation. Hamster blood was collected heparinized from cardiac puncture and immediately injected intraperitoneally into rats. Pig blood was obtained heparinized from animals housed at the Vall d'Hebron Research Institute (Barcelona, Spain).

Determination of Xenoantibodies

IgM and IgG xenoantibodies were determined by flow cytometry. Target cells included lymphocytes obtained from hamster, rabbit and rat spleen, and porcine and human cell lines from the European Collection of Cell Cultures. These consisted of pig lymphoblast (L35), porcine aortic endothelial cells (PAEC; P304-05), human T-lymphoblastic cells (Jurkat) and human microvascular endothelial cells (HUMEC). The rat endothelial cell line LEW-1A was a gift from Dr. Ignacio Anegón (INSERM UMR 643, Nantes, France). Target cells (1×10^6 cells per sample) were incubated with test sera diluted 1/50 in PBS/1% bovine serum albumin (BSA) at 4°C for 30 min in a final volume of 100 μ l in V-bottom 96-well microtiter plates (Nunc Denmark). After first incubation, a wash with PBS/1% BSA was performed followed by a second incubation (4°C for 30 min in the dark) with 100 μ l of a mixture of two polyclonal secondary antibodies – goat F(ab')₂ fragment anti-rat IgG (H+L) conjugated with dichlorotriazinyl aminofluorescein (dilution 1/100) and goat F(ab')₂ fragment anti-rat IgM (μ) conjugated with phycoerythrin (dilution 1/200; Beckman Coulter™ Immuno-tech) in PBS/1% BSA. Finally, cells were washed, resuspended in PBS and transferred to FACS tubes. For the determination of fluorescence intensity, a FACScalibur cytometer (BD Biosciences) was used with three detectors/photomultipliers which detect light emitted at 530 (FL1), 585 (FL2) and >670 nm (FL3), along with the help of programs for acquisition and analysis (Cell Quest) as well as for verification (FACS Comp).

Reactivity of Xenoantibodies with Bacteria

Cross-reactivity of xenoantibodies was assessed with *E. coli* and *Enterococcus faecalis* isolated from Lewis rat blood sampling 24 h after cecal ligation and puncture (CLP) sepsis. Bacteria were isolated after incubating 1–100 µl of blood in a blood agar (base; Sigma-Aldrich, Spain) plate for 24 h at 37°C. In addition, xenoantibody cross-reactivity was also evaluated with *E. coli* and two different *E. faecalis* isolated from human blood, provided by the Microbiology Department from Bellvitge University Hospital. A cell suspension of 100 µl of *E. coli* and *E. faecalis* kept frozen in glycerine was incubated in 5 ml Luria-Bertoni medium for 12 h at 37°C under continuous shaking until the culture reached saturation. Then, 100 µl from these saturated cultures were inoculated in 5 ml of fresh Luria-Bertoni medium and incubated again on a shaker at 37°C until reaching an optical density of 0.3 at 600 nm determined with a spectrophotometer Ultrospec 2000 (Pharmacia Biotech, Cambridge, UK). At this point, bacteria were centrifuged at 3,000 rpm for 5 min at 4°C, supernatant aspirated and resuspended in 2.5 ml of PBS/1% BSA. Rat serum samples in a 1/500 dilution were incubated at 4°C for 30 min with bacteria. Subsequently, a second incubation (at 4°C for 30 min) with secondary anti-rat antibodies was performed followed by flow cytometric analysis, as described for determination of xenoantibodies.

Glycan Array Analysis

The glycan microarrays from the Consortium for Functional Glycomics were prepared from amine-functionalized glycan structures covalently coupled in microarrays to N-hydroxysuccinimide-derivatized microscope slides as previously described [14]. Glycan reactivity of rat IgM and IgG xenoantibodies was analyzed for binding to version 4.2 of the printed array that included 511 glycan targets (Consortium for Functional Glycomics Project No. 2115). A complete glycan listing and their identification numbers can be found at <http://www.functionalglycomics.org/static/consortium/resources/resourcecoreh15.shtml>.

Inhibition Assays

The specificity of xenoantibody binding to *E. faecalis* or xenogenic cells was studied using a competitive assay with L-rhamnose, melibiose, Forssman antigen disaccharide or N-acetylneuraminic acid (Sigma-Aldrich, Spain), as well as with GlcAβ1-6Galβ, Galα1-4GlcNAcβ and GalNAcβ1-6GalNAcβ (kindly provided by Prof. Nicolai Bovin, Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Science, Moscow, Russian Federation). Rat serum diluted 1/500 (*E. faecalis*) or 1/50 (xenogenic cells) was initially incubated with different concentrations of carbohydrates at 4°C for 30 min, followed by a second incubation with *E. faecalis* or xenogenic cells at 4°C for 30 min. Rat secondary antibody incubation and fluorescence analysis was performed by FACS as described.

Cecal Ligation and Puncture

CLP was performed as described elsewhere [15]. Briefly, Lewis rats were anesthetized with 3–4% isoflurane, and under sterile conditions, a 1- to 2-cm midline incision was made and the cecum was exteriorized and ligated (4-0 Safil® Violet, B. Braun, Germany) distal to the ileocecal valve. For low-grade sepsis, defined as approximately 0–15% of mortality during the acute phase of sepsis, 25% of cecum (approx. 10 mm) was ligated and punctured twice with a 19-gauge needle. The abdomen was closed in two layers, and

animal recovery was facilitated keeping the animal on a thermal blanket. A subcutaneous injection of 1 ml of dextrose and analgesia with buprenorphine (0.05 mg/kg) was given every 8 h up to 7 days.

Statistical Analysis

Results are expressed as the mean ± standard error of the mean (SEM). Differences between groups were compared by the unpaired Student t test. Survival data were compared by the log-rank test. Results were considered statistically significant if $p < 0.05$.

Results

Pattern of IgM and IgG Xenoantibodies in Rats after Hamster and Pig Blood Immunization

Enhancement of TI xenoantibodies implied a rapid increase in anti-hamster and anti-pig IgM from day 0 to 5, peaking in both cases at the latter day (fig. 1a, c), compared to levels existing before immunization and at day 5 in C-TI rats. Thereafter, anti-hamster and anti-pig IgM dropped; the levels at day 20 were still elevated for anti-hamster but not for anti-pig xenoantibodies compared to those present at baseline and maintained during all the study in the C-TI rats. At day 5, TI xenoantibodies also included anti-hamster and anti-pig IgG that were barely half the level of IgM antibodies (fig. 1a, c). Production of rat TD xenoantibodies was associated with a marked increase in anti-hamster and anti-pig IgG compared to pre-immune samples which was not observed in C-TD rats (fig. 1b, d). The highest level of TD IgG was observed on days 40 and 55 for anti-hamster and anti-pig xenoantibodies, respectively. There was also some increase in anti-hamster and anti-pig IgM at day 40, which was 4- and 6-fold lower than the IgG xenoantibodies, respectively (fig. 1b, d).

Cross-Reactivity of IgM and IgG Anti-Hamster and Anti-Pig Xenoantibodies with Bacteria

Reactivity of Lewis rat TI and TD anti-hamster and anti-pig IgM and IgG xenoantibodies on days 5 (C-TI and TI) and 40 (C-TD and TD) was evaluated with *E. coli* and *E. faecalis* isolated from Lewis rat blood after CLP (data not shown). C-TI and C-TD, as well as TI and TD xenoantibodies, did not show any reactivity with *E. coli* either for IgM and IgG isotypes (fig. 2). In contrast, C-TI and C-TD IgM antibodies evidenced some reactivity with *E. faecalis* that significantly increased with TI anti-hamster and anti-pig antibodies (fig. 2a, c). In addition, TI IgG xenoantibody reactivity to *E. faecalis* significantly increased with anti-hamster and anti-pig antibodies (fig. 2).

Fig. 1. Generation of rat anti-hamster and anti-porcine xenoantibodies. Pattern of TI anti-hamster (a) and anti-porcine (c) xenoantibodies in Lewis rats with 3 injections every other day of hamster or pig blood, respectively. Pattern of TD anti-hamster (b) and anti-porcine (d) xenoantibodies in Lewis rats with 3 injections every other week of hamster and pig blood, respectively. C-TI and C-TD groups received PBS on the same days that TI and TD animals were injected with xenogenic blood. Results show the mean $\pm$ SEM of 4 animals for each type of immunization and protocol. * $p < 0.05$, TI versus C-TI and TD versus C-TD. MFI = Mean fluorescence intensity.

Generation of TD anti-hamster and anti-pig IgM and IgG was not associated with any change in reactivity to *E. faecalis* (fig. 2).

Reactivity of Lewis rat TI anti-hamster and anti-pig IgM and IgG xenoantibodies on day 5 was also evaluated with one *E. coli* and two different *E. faecalis* isolated from human blood. Only boosted anti-pig IgM xenoantibodies showed a statistically significant increase in binding to the two *E. faecalis* isolates compared to baseline (fig. 2). However, the reactivity was much lower than that observed with *E. faecalis* isolated from Lewis rat blood.

Cross-Reactivity of IgM and IgG Anti-Hamster and Anti-Pig Xenoantibodies with Other Species

Reactivity of Lewis rat TI and TD anti-hamster and anti-pig IgM and IgG xenoantibodies on days 5 (C-TI and TI) and 40 (C-TD and TD) was evaluated with lympho-

cytes and endothelial cells from other species. C-TI and C-TD IgM and IgG antibodies showed some reactivity to hamster lymphocytes, porcine lymphoblasts (L35), human T-lymphocytes (Jurkat), rabbit lymphocytes, and rat lymphocytes (fig. 3). TI and TD anti-hamster IgM and IgG xenoantibodies, besides targeting hamster lymphocytes, also cross-reacted with porcine lymphoblasts, human T lymphocytes and rabbit lymphocytes, but not with rat lymphocytes (fig. 3a, b). TI IgM anti-porcine xenoantibodies cross-reacted with hamster or rabbit lymphocytes and human Jurkat cells but not with rat lymphocytes (fig. 3c, d). TD IgM anti-pig xenoantibodies exhibited a similar pattern of cross-reactivity with the exception of rabbit lymphocytes. TI IgG anti-pig xenoantibodies also targeted hamster lymphocytes, whilst TD IgG bound hamster lymphocytes and Jurkat cells (fig. 3c, d). We also investigated whether rat xenoantibodies cross-reacted

Fig. 2. Reactivity of xenoantibodies with rat and human *E. faecalis* and *E. coli*. Anti-hamster IgM xenoantibodies (a); anti-hamster IgG xenoantibodies (b); anti-pig IgM xenoantibodies (c); anti-pig IgG xenoantibodies (d). Results show the mean $\pm$ SEM of 4 animals for each type of immunization and protocol of xenoantibody production. * $p < 0.05$, TI versus C-TI. Reactivity against only one of the two human *E. faecalis* tested is shown. MFI = Mean fluorescence intensity; XAb = xenoantibodies.

with xenogeneic endothelial cells. C-TI and C-TD IgM antibodies showed some reactivity to PAEC, human microvascular endothelial cells and rat endothelial cells (fig. 4). TI anti-hamster IgM as well as TI and TD anti-porcine IgM or IgG xenoantibodies showed a statistically significant augment of binding to HMEC but not to rat endothelial cells (fig. 4a–d). Anti-hamster xenoantibodies did not show any reactivity to PAEC (fig. 4a–d).

Anti-Carbohydrate Antibody Repertoire after Xenogeneic Immunization in Rat

Glycan reactivity of rat TI IgM and IgG xenoantibodies was analyzed for binding to a printed array that included 511 glycan targets. Before xenogenic exposure, no carbohydrate was bound by rat IgM natural antibodies with a mean reactivity $>1,000$ relative fluorescence units (RFU), although there were three binding with IgG antibodies. These included L-rhamnose (Rha α ; glycan No. 8), melibiose (Gala1-6Glc β ; glycan No. 122) and GlcA β 1-6Gal β (glycan No. 201; fig. 5, 6). Boosted rat TI anti-hamster xe-

noantibodies targeted 1 carbohydrate (L-rhamnose) with a mean reactivity $>1,000$ RFU for IgM and 10 with IgG antibodies (fig. 5a, 6a). The generation of TI anti-pig xenoantibodies was associated with a much higher reactivity against carbohydrates than with anti-hamster antibodies. There were 6 carbohydrates bound by IgM and 65 by IgG anti-pig antibodies, with a mean reactivity of $>1,000$ RFU (fig. 5b, 6b). The reactivity of IgM antibodies with carbohydrates was lower than that of IgG, but the pattern of recognition of the top 20 glycans was similar to the two Ig isotypes for both anti-hamster and anti-pig antibodies (fig. 5, 6). The top 20 carbohydrates targeted by IgM included 3 carbohydrates recognized by both anti-hamster and anti-pig antibodies. These were L-rhamnose, GlcA β 1-6Gal β and complex glycan No. 372 with terminal Fuca1-2Gal β (fig. 5a, b). Among the top 20 carbohydrates bound by IgG, there were 5 targeted by both anti-hamster and anti-pig antibodies. These included L-rhamnose, melibiose, GlcA β 1-6Gal β , GalNAc β 1-6GalNAc β (glycan No. 440) and Gal β 1-4(6OSO3)Glc β (glycan No. 153).

Fig. 3. Reactivity of xenoantibodies with hamster, porcine, human, rabbit and rat lymphocytes. Anti-hamster IgM xenoantibodies (a); anti-hamster IgG xenoantibodies (b); anti-pig IgM xenoantibodies (c); anti-pig IgG xenoantibodies (d). Results show the mean $\pm$ SEM of 4 animals for each type of immunization and protocol of xenoantibody production. * $p < 0.05$, TI versus C-TI and TD versus C-TD. MFI = Mean fluorescence intensity; XAb = xenoantibodies.

Fig. 4. Reactivity of xenoantibodies with porcine, human and rat endothelial cells. Anti-hamster IgM xenoantibodies (a); anti-hamster IgG xenoantibodies (b); anti-pig IgM xenoantibodies (c); anti-pig IgG xenoantibodies (d). Results show the mean $\pm$ SEM of 4 animals for each type of immunization and protocol of xenoantibody production. * $p < 0.05$, TI versus C-TI and TD versus C-TD. MFI = Mean fluorescence intensity; XAb = xenoantibodies.

Fig. 5. Glycan-binding pattern of anti-hamster and anti-pig IgM xenoantibodies. Top 20 glycans bound by TI IgM anti-hamster (**a**) and anti-pig (**b**) xenoantibodies, along with the reactivity of these carbohydrates in the same animals before xenoimmunization. The

results show the mean $\pm$ SEM of two experiments for each type of xenoimmunization. The full glycan structure is shown at <http://www.functionalglycomics.org/static/consortium/resources/resourcecoreh15.shtml>.

Inhibition of Xenoantibody Cross-Reactivity with Carbohydrates

In order to evaluate the role of carbohydrate antigens in rat TI anti-hamster and anti-porcine xenoantibody cross-reactivity with *E. faecalis* and cells from other species, we performed competitive assays with L-rhamnose, melibiose, GlcA β 1-6Gal β , and GalNAc β 1-6GalNAc β , which were recognized by both anti-hamster and anti-pig xenoantibodies. Also, competitive assays were done with Forssman antigen disaccharide (GalNAc α 1-3GalNAc), N-acetylneuraminic acid (Neu5Ac β) and Gal α 1-4GlcNAc β , which were terminal elements of oligosaccharides targeted with a high level of reactivity by IgM and IgG anti-porcine antibodies. Melibiose inhibited the rat xenoantibody reactivity to *E. faecalis* in a concentration-dependent manner. At 20 mM melibiose, anti-hamster IgM and IgG reactivity was 36 and 25% of the original antibody binding (fig. 7a, b), and for anti-porcine IgM and IgG antibodies, 77 and 76% (fig. 7c, d), respectively.

L-Rhamnose also showed some inhibition of reactivity which at 20 mM was 26 and 22% of the initial antibody binding for anti-hamster IgM and IgG, respectively (fig. 7a, b), and 12 and 15% for anti-porcine IgM and IgG (fig. 7c, d). Exclusively for anti-pig xenoantibodies, N-acetylneuramic acid exhibited a small inhibition of reactivity (8% for IgM and 18% for IgG at 20 mM; fig. 7c, d). None of the other carbohydrates tested demonstrated any inhibition of rat xenoantibody reactivity to *E. faecalis*. Competitive assays assessing the cross-reactivity of rat anti-hamster and anti-pig xenoantibodies with porcine (L35) or hamster lymphocytes, Jurkat cells, rabbit lymphocytes and HMEC did not show any inhibition with the 7 carbohydrates tested (data not shown).

Impact of Anti-Hamster and Anti-Pig Xenoantibodies in Lewis Rat Survival after CLP

CLP for low-grade sepsis was performed in Lewis rats on day 5 after generation of TI xenoantibodies and cor-

Fig. 6. Glycan-binding pattern of anti-hamster and anti-pig IgG xenoantibodies. Top 20 glycans bound by TI IgG anti-hamster (**a**) and anti-pig (**b**) xenoantibodies, along with the reactivity of these carbohydrates in the same animals before xenoimmunization. The

results show the mean $\pm$ SEM of two experiments for each type of xenoimmunization. The full glycan structure is shown at <http://www.functionalglycomics.org/static/consortium/resources/resourcecoreh15.shtml>.

responding C-TI, and on day 40 after production of TD xenoantibodies or C-TD. The rise in TI xenoantibodies was associated with a decreased survival after CLP, which was 52.9 and 60% with anti-hamster ($n = 17$) and anti-pig antibodies ($n = 10$), respectively, compared to 88.2% in C-TI ($n = 17$; fig. 8). The difference was statistically significant for the enhancement of anti-hamster antibodies ($p < 0.05$, as determined by log-rank survival analysis). Generation of TD xenoantibodies did not modify rat survival in low-grade sepsis, which was in all cases 100% (data not shown).

Discussion

Natural xenoantibodies, considered to be directed against microorganisms, cross-react with antigens, also exhibited by cells from other species [3]. These antibodies have gained attention due to their role in the rejection of grafts transplanted between different species as a consequence of their cross-reactivity. The work reported here was aimed to characterize the recognition of microorgan-

isms by xenoantibodies in Lewis rats. We took advantage of the well-defined antibody response of these rats to xenoantigens to boost TI (natural) and TD (adaptive) xenoantibodies and examined their reactivity to bacterial antigens [12]. Rat natural IgM antibodies showed some reactivity to *E. faecalis* isolated from the blood of Lewis rats after CLP. This reactivity was significantly enhanced with the increase in TI but not in TD IgM and IgG anti-hamster and anti-pig xenoantibodies. Boosted TI IgM anti-pig xenoantibodies also showed some binding to two distinct *E. faecalis* isolates from human blood, substantiating that the cross-reactivity was related to TI antigens. In contrast, both natural and boosted TI rat xenoantibodies did not exhibit any reactivity to rat or human *E. coli*. The existence of some natural IgM antibodies reactive to *E. faecalis* before xenogenic exposure, and the lack of reactivity to *E. coli*, may explain the TI responses observed against these microorganisms after immunization. If hamster and pig blood contain antigens targeted by natural antibodies, which apparently are also present in *E. faecalis*, a rapid production of more antibodies against these antigens will occur. In addition, antibodies targeting oth-

Fig. 7. Inhibition of xenoantibody binding to *E. faecalis* by oligosaccharides. Competitive assay of TI anti-hamster IgM (a) and IgG (b) or anti-pig IgM (c) and IgG (d) xenoantibodies reactive to *E. faecalis* by preincubation of sera with 7 different oligosaccharides. The results show the percentage of the average inhibition produced by each oligosaccharide at different concentrations (n = 4).

Fig. 8. Survival of Lewis rats after CLP following the generation of TI anti-hamster (n = 17) and anti-pig (n = 10) xenoantibodies (XAb) or PBS injections (C-TI; n = 17). * p < 0.05.

er *E. faecalis* antigens may be produced. It has been previously shown that natural IgM antibodies reacting to α Gal antigens play a key role in increasing the efficiency of priming to other antigens conjugated with α Gal without the need for any adjuvant [16]. In contrast, immunization in the absence of anti- α Gal IgM antibodies failed to induce reactive antibodies against the non- α Gal antigens bound to α Gal. Therefore, the lack of natural IgM antibodies reactive to *E. coli* may result in the absence of the production of cross-reactive TI xenoantibodies, even if hamster and pig blood share some antigens with the microorganism.

The reactivity of Lewis rat TI xenoantibodies to *E. faecalis* was enhanced by exposure to both closely (hamster) and distantly related (pig) animal species. In addition, rat natural antibodies as well as TI and TD IgM and IgG anti-hamster and anti-porcine xenoantibodies reacted to

lymphocytes and endothelial cells of closely and distantly related animal species. The cross-reactivity of TI and TD rat xenoantibodies from closely related species with other closely related species was previously shown, both by binding to cells of these species and by mediating graft rejection in xenotransplantation experiments [17]. In contrast, cross-reactivity of rat xenoantibodies with distantly related species was not evidenced in transplant experiments [18], though it was observed in mice and rabbits after injection of xenogenic endothelial cells [19, 20]. Rat natural IgM and IgG antibody reactivity to lymphocytes from closely and distantly related species may result from the binding of antibodies to Fc receptors on these cells. This could also explain the higher reactivity to hamster compared to pig lymphocytes observed after exposure to pig blood. This type of reactivity makes the assessment of antigens targeted by enhanced xenoantibodies with these cells very difficult. However, the reactivity of rat anti-hamster and anti-pig xenoantibodies with cells from other species also included long-lasting TD xenoantibodies. These xenoantibodies were not observed in reactivity to *E. faecalis*, suggesting that antibodies to cells from other species target distinct antigens. In addition, TD rat anti-pig xenoantibodies showed a particular reactivity to HMEC. There is also evidence of a similar response to HMEC after immunizing non-human primates with porcine red blood cells [unpublished], suggesting that the latter cells express antigens that are specifically shared with HMEC. The work reported here does not allow definition of the particular role of blood cellular elements in the generation of TI and TD xenoantibodies. However, previous data suggest that erythrocytes may be mainly involved in the production of TI and leukocytes in TD xenoantibodies [21, 22].

Natural antibodies are primarily IgM and mainly target carbohydrate antigens. In our glycan array data, the TI IgM reactivity to carbohydrates was much lower than that of IgG, both before and after xenoimmunization with hamster and porcine blood. Disparity may be due to the competition of both antibody isotypes for the same antigen in the glycan array and a higher affinity of anti-glycan IgG compared to IgM, giving rise to more intense signals with the former antibodies [23]. Melibiose, L-rhamnose and GlcA β 1-6Gal β reactivity were present in preimmunization samples and showed a substantial augment with boosted TI anti-hamster and anti-pig xenoantibodies. Melibiose and L-rhamnose, but not GlcA β 1-6Gal β , were able to inhibit antibody binding to *E. faecalis*, suggesting the involvement of the 2 former carbohydrates in the reactivity of xenoantibodies to the

bacterium. Melibiose showed the strongest inhibition on the binding of rat anti-pig xenoantibodies to *E. faecalis*, whilst there was less inhibition of anti-hamster reactivity, similar to that exhibited by L-rhamnose for these xenoantibodies. No carbohydrate tested inhibited the cross-reactivity of anti-hamster and anti-porcine xenoantibodies with cells from other species, confirming that the antigens involved in this reaction are distinct from those targeting *E. faecalis*. Identification of carbohydrate antigens expressed on cells and involved in the rejection of hamster xenografts in rats has remained elusive despite substantiating significant changes like with anti-Forssman antigen antibodies [24]. We cannot rule out that most of the cross-reactivity of xenoantibodies with cells from other species is due to a polyreactivity to the structural nature of the antigen-binding site rather than to antigenic similarity [25, 26].

Anti-melibiose antibodies are considered anti- α -galactosyl antibodies in mammals similar to anti- α Gal in humans and closely related primates [27, 28] who lack the α 1,3-galactosyltransferase gene and α Gal antigen [29]. Bovine anti-melibiose antibodies react with the type II bovine strain of *Streptococcus agalactiae*, a common pathogen of bovine mastitis, but not with human type IV and V strains that do not express melibiose [30]. Humans also exhibit high titers of natural anti-melibiose antibodies [31], which may be related to host-bacterial interactions with some strains of *E. faecalis* or other *Streptococcus* spp. that display this carbohydrate antigen in the cell wall [32, 33]. There is very little information available on cell surface-associated polysaccharides of enterococci. Crude cell wall extracts from *E. faecalis* type 1 strains performed in the 1960s were found to contain L-rhamnose, glucose, galactose, N-acetylglucosamine and N-acetyl-galactosamine [34]. More recently, a carbohydrate polymer was identified containing L-rhamnose, glucosamine, galactosamine, glucose, galactose, and phosphate in a 4:2:2:1:1:1 ratio [35]. In addition, the presence of L-rhamnose in the *E. faecalis* cell wall may be predicted by the presence of the OG1RF gene, which is involved in the synthesis of thymidine diphosphate rhamnose, a precursor from which L-rhamnose can be transferred to polysaccharides [36, 37]. However, L-rhamnose was considered non-antigenic despite being present in the cell wall, because it was absent from hydrolysates of purified serologically active fractions of human isolates of *E. faecalis* that included melibiose [32]. Our studies cannot elucidate whether L-rhamnose on the *E. faecalis* wall is antigenic for rats since the reactive antibodies were generated by xenoantigens and not by the bacterium.

The role of TI natural antibodies in the defense against infections has been defined by passive transfer experiments in animal models lacking these antibodies [38]. However, there is also evidence that in some conditions, TI natural anti-carbohydrate antibodies may enhance the infective potential of microorganisms instead of being protective to the host [7, 39]. We previously showed that elevated natural anti- α Gal IgM levels at the start of dialysis were predictors of later risk for mortality and enteric peritonitis in hemodialysis and peritoneal dialysis patients, respectively [40]. In the present study, the rise in TI anti-hamster and anti-pig xenoantibodies that reacted to *E. faecalis* by targeting melibiose and L-rhamnose was associated with an increased mortality of rats after CLP. Human sera, as shown in this study with rat sera, contain natural antibodies against *E. faecalis* that promote neutrophil-mediated killing by complement activation. However, these natural antibodies do not protect against infection by *E. faecalis* [41, 42]. One of the reasons is that the microorganism produces capsular polysaccharides that contribute to the pathogenesis by making it more resistant to complement-mediated opsonophagocytosis [43]. Natural antibodies such as anti- α Gal have shown the ability to also interfere with complement, generating

Gram-negative bacteria strains, with a particular capacity for spreading to the blood [7, 44]. Whether the lack of a protective effect of natural antibodies against *E. faecalis* moves to a deleterious effect if these antibodies are boosted, impairing the survival of the rat after CLP, is unclear and requires further investigations.

In summary, rat TI xenoantibodies include antibodies against melibiose and L-rhamnose reactive to *E. faecalis*. Enhancement of these antibodies may impair the infection caused by microorganisms of endogenous flora, suggesting that modulation of natural antibodies may represent a new therapeutic strategy for bacterial diseases and that xenogenic immunization may help to characterize those antibodies involved in the reaction.

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